MED-MEDICAL
OPEN SHELF
CURRENT

THE AUSTRALIAN JOURNAL OF EXPERIMENTAL BIOLOGY AND MEDICAL SCIENCE

EDITOR-IN-CHIEF
MARK L. MITCHELL

CO-EDITORS
G. M. BADGER
D. I. B. KERR

UNIVERSITY OF
RECEIVED
1962

VOL. XXXIX

PUBLISHED BY THE UNIVERSITY OF ADELAIDE

PRINTED AT THE GRIFFIN PRESS, ADVERTISER LANE, ADELAIDE
1961

U of M Bindery NOV 15 52 (8)

B570.5
Au78

CONTENTS

PART 1. FEBRUARY, 1961

PAGE

W. R. PITNEY AND P. ONESTI: Vitamin B_{12} and folic acid concentrations of human liver with reference to the assay of needle biopsy material - 1

J. B. EGAN AND B. W. HOLLOWAY: Genetic studies in lysogeny in *Pseudomonas aeruginosa* - - - - - - - - - - - - 9

J. K. A. CLEZY, BARBARA J. DENNIS, AND D. I. B. KERR: A degeneration study of the somaesthetic afferent systems in the marsupial phalanger, *Trichosurus vulpecula* - - - - - - - - - - - 19

BARBARA J. DENNIS AND D. I. B. KERR: Somaesthetic pathways in the marsupial phalanger, *Trichosurus vulpecula* - - - - - - - - 29

BARBARA J. DENNIS AND D. I. B. KERR: An afferent system in the central tegmental fasciculus - - - - - - - - - - - - - 43

EARLE HACKETT AND REX LEPAGE: The clotting of the blood of an amphibian, *Bufo marinus* Linn. 1. Prothrombin-thrombin and "fibrinogen-fibrin" stages - - - - - - - - - - - - 57

EARLE HACKETT AND REX LEPAGE: The clotting of the blood of an amphibian, *Bufo marinus* Linn. 2. Blood thromboplastic activity - 67

MARGARET RYLE AND L. R. MORRIS: Some quantitative studies on tissues of lambs dwarfed by high temperatures during gestation - - - - 79

JEAN YOUATT: Pigments produced by *Mycobacteria* on exposure to isoniazid - - - - - - - - - - - - - - - - 93

DEBORAH BURNET AND F. M. BURNET: Analysis of major histocompatibility factors in a stock of closely inbred white leghorn fowls using a graft-*versus*-host reaction on the chorioallantoic membrane - - - - 101

U of M Bindery NOV 15 52

PART 2. APRIL, 1961

PAGE

M. SOMERS: Factors influencing the secretion of nitrogen in sheep saliva. 1. The distribution of nitrogen in the mixed and parotid saliva of sheep - - - - - - - - - - - - - - - 111

M. SOMERS: Factors influencing the secretion of nitrogen in sheep saliva. 2. The influence of nitrogen intake upon blood urea nitrogen and upon the total nitrogen and urea nitrogen in the parotid saliva of sheep 123

M. SOMERS: Factors influencing the secretion of nitrogen in sheep saliva. 3. Factors affecting the nitrogen fractions in the parotid saliva of sheep with special reference to the influence of ammonia production in the rumen and fluctuations in level of blood urea - - - - - 133

M. SOMERS: Factors influencing the secretion of nitrogen in sheep saliva. 4. The influence of injected urea on the quantitative recovery of urea in the parotid saliva and the urinary excretions of sheep - - - 145

G. T. ARCHER AND NOLA BOSWORTH: Phagocytosis by eosinophils following antigen-antibody reactions *in vitro* - - - - - - - - - 157

NOLA BOSWORTH AND G. T. ARCHER: The eosinophil content of the peritoneal cavity of the rat - - - - - - - - - - - 165

F. GIBSON AND BARBARA MCDOUGALL: The effect of chloramphenicol and oxytetracycline on the formation of intermediates in tryptophan biosynthesis - - - - - - - - - - - - - - 171

EDWARD A. JOHNSON, J. TILLE AND LESLEY WILSON: The effect of a change in shape of the cardiac action potential on the ionic conductances - 179

V. ELIZABETH HODGETTS: The dynamic red cell storage function of the spleen in sheep. III. Relationship to determination of blood volume, total red cell volume, and plasma volume - - - - - - - - 187

C. C. CURTAIN: The analysis of electrophoresis patterns: a comparison of two methods with the aid of Rayleigh interference optics - - 197

C. C. CURTAIN: The quantitative electrophoresis of serum proteins in the presence of haemoglobin - - - - - - - - - - - 203

PART 3. JUNE, 1961

PAGE

W. D. BROWN: Errors in the determination of serum cholesterol - - 209

W. D. BROWN: A filtration procedure for determining serum cholesterol with digitonin - - - - - - - - - - - - - 223

N. L. WARNER AND F. M. BURNET: The influence of anti-inflammatory corticosteroids on the growth of the chick embryo and the manifestations of the Simonsen phenomenon - - - - - - - - - 235

J. MARGOLIS: The effect of colloidal silica on blood coagulation - - - 249

E. A. NORTH, G. PAWLYSZYN AND HAZEL M. DOERY: The action of phosphatidase A, sodium oleate and ganglioside on the exotoxins of *Cl. welchii* and on the neurotoxin of *Shigella shigae* - - - - - 259

E. P. GEORGE: Resistance values in a syncytium - - - - - - 267

E. P. GEORGE AND E. A. JOHNSON: Solutions of the Hodgkin-Huxley equations for squid axon treated with tetraethylammonium and in potassium-rich media - - - - - - - - - - - - 275

D. I. ANNEAR: Recovery of *Strigomonas oncopelti* after drying from the liquid state - - - - - - - - - - - - - - 295

PART 4. AUGUST, 1961

PAGE

MARY C. DAWBARN, HEATHER FORSYTH AND DIANA KILPATRICK: Some factors which affect the growth of *Lactobacillus casei* - - - 305

C. H. GALLAGHER: The effect of hepatotoxins on E_{260} values of liver and of serum - - - - - - - - - - - - - - 323

HAZEL M. DOERY AND E. A. NORTH: The interaction of staphylococcal toxin and ganglioside. 1. Inactivation of the lethal effect of staphylococcal toxin in mice - - - - - - - - - - - 333

B. T. BROWN, W. S. GOLDER AND S. E. WRIGHT: The interconversion of oestrone and oestradiol-17β by human and rat blood - - - - - 345

A. R. JOHNSON AND F. R. HEWGILL: The effect of the antioxidants, butylated hydroxy anisole, butylated hydroxy toluene and propyl gallate on growth, liver and serum lipids and serum sodium levels of the rat - 353

E. H. MORGAN: Iron storage and transport after administration of viable erythrocytes to the albino rat - - - - - - - - - 361

E. H. MORGAN: Iron storage and transport in iron-depleted rats, with notes on combined iron and copper deficiency - - - - - - 371

R. J. WALSH AND SHIRLEY CANTRILL: The mechanism of absorption of subcutaneous extravasations of blood - - - - - - - - 381

C. C. CURTAIN: Localization of 18 S gamma-macroglobulin in plasmacytoid cells of the bone marrow of a case of macroglobulinaemia - - - 391

PART 5. OCTOBER, 1961

PAGE

R. E. Davis: Folic acid deficiency and urinary formiminoglutamic acid excretion in the rat - - - - - - - - - - - - - 397

P. Fantl: A comparative study of blood coagulation in vertebrates - - 403

Stephen Fisher: Observations on an anti-staphylococcal mouse protective antibody in human sera - - - - - - - - - - - - 413

B. L. Hillcoat: The excretion of β-aminoisobutyric acid following thymine and dihydrothymine administration to man - - - - - - 423

Winifred G. Nayler: Changes in cardiac contractility evoked under isotonic and isometric conditions - - - - - - - - - 429

D. Metcalf, N. Sparrow, K. Nakamura and M. Ishidate: The behaviour of thymus grafts in high and low leukaemia strains of mice - - 441

R. G. Wales, T. W. Scott and I. G. White: Biuret reactive materials in semen - - - - - - - - - - - - - - - 455

D. S. Roberts: The life cycle of *Dermatophilus dermatonomus*, the causal agent of ovine mycotic dermatitis - - - - - - - - - 463

J. R. Thayer and G. C. Owen: An improved method for aseptic ultrafiltration - - - - - - - - - - - - - - - 477

PART 6. DECEMBER, 1961

PAGE

LORNA M. A. GREEN: Sweat glands in the skin of the quokka of Western Australia - - - - - - - - - - - - - - - 481

J. K. DINEEN AND ALEKSANDER SZENBERG: The evidence for the survival and and regeneration of donor epithelial elements in orthotopically-grafted skin homo-transplants - - - - - - - - - - - 487

PATRICIA E. LIND AND A. SZENBERG: Quantitative aspects of the Simonsen phenomenon. III. The effect of immunization of the donor fowl - 507

C. A. PARKER: A method for the measurement of total nitrogen in microgram amounts - - - - - - - - - - - - - 515

J. H. POPE: Studies of a virus isolated from a wild house mouse, *Mus musculus*, and producing splenomegaly and lymph node enlargement in mice - - - - - - - - - - - - - - - 521

R. FOWLER, JNR., AND C. D. WEST: Further evidence against the graft-versus-host hypothesis in renal transplantation - - - - - - 537

S. FAZEKAS DE ST.GROTH AND R. G. WEBSTER: Methods in immunochemistry of viruses. I. Equilibrium filtration - - - - - - - - 549

S. FAZEKAS DE ST.GROTH: Methods in immunochemistry of viruses. 2. Evaluation of parameters from equilibrium measurements - - 563

G. WOOLLEY: Effects of morphine sulphate on capillary filtration and thoracic duct lymph formation in the rat - - - - - - - 583

H. F. HOLDEN AND K. LYNIKAS: The production of green pigments from the blood pigment - - - - - - - - - - - - 595

L. J. OPIT AND J. P. SAVAGE: Glucose metabolism in human (Thiersch) skin grafts - - - - - - - - - - - - - - 601

J. V. HURLEY AND N. XEROS: Electron microscopic observations on the emigration of leucocytes - - - - - - - - - - - 609

VITAMIN B_{12} AND FOLIC ACID CONCENTRATIONS OF HUMAN LIVER WITH REFERENCE TO THE ASSAY OF NEEDLE BIOPSY MATERIAL

by W. R. PITNEY AND P. ONESTI

(From the Department of Haematology, Royal Perth Hospital, Western Australia).

(*Accepted for publication 5th December, 1960.*)

SUMMARY.

Vitamin B_{12} and folic acid concentrations in samples of human liver have been assayed microbiologically. The yield of both vitamins is increased by papain digestion of liver homogenates. The vitamin B_{12} concentrations of ten normal livers ranged from 0·61 μg./gm. to 1·02 μg./gm. wet tissue; the folic acid concentrations of the same livers ranged from 0·69 μg./gm. to 1·60 μg./gm. wet tissue.

Material aspirated from the liver by suction needle biopsy was satisfactory for the assay of vitamin B_{12} and folic acid provided that the specimen weighed at least 7 mg.

Methods are described for the processing and assay of needle biopsy material.

INTRODUCTION

The histological examination of material obtained by percutaneous needle biopsy of the liver is an established procedure. The purpose of the present investigation was to determine the degree of accuracy with which microbiological assays of vitamin B_{12} and folic acid could be performed on needle biopsy material. Such specimens are small, often fragmented and difficult to weigh and process accurately. They are not as satisfactory to handle as samples of liver obtained by surgical biopsy. Nevertheless, vitamin B_{12} concentrations have been reported from needle biopsy material (Drouet, Wolff, Karlin-Weissman and Rauber, 1951; Nelson and Doctor, 1958; Jhala and Gadgil, 1960) and clinical significance has been attached to the results. In this paper assay values for needle biopsy specimens are compared with those obtained from blocks of tissue from the same livers. A method for processing needle biopsy material is described and values are given for the vitamin B_{12} and folic acid concentrations of normal liver samples.

MATERIAL.

Assays were carried out on liver samples from two groups of subjects. The first group were ten previously healthy individuals who had died suddenly. A block of tissue weighing approximately 5 gm. was removed from the right lobe of each liver at autopsy. This material

was used to test methods of extraction of vitamin B_{12} and folic acid and to obtain a normal range of values for these vitamins.

The second group were 12 hospital patients dying from various causes. Two (Cases 11 and 22) suffered from hepatic cirrhosis. At autopsy blocks of tissue were removed from both the right and left lobes of the liver and a needle biopsy sample using a Franseen needle was obtained from the right lobe. The three samples from each were assayed for vitamin B_{12} and folic acid. A specimen from each liver was also examined histologically.

METHODS.

Needle biopsy material.

The biopsy specimen was removed from the syringe, laid on filter paper to remove excess saline and weighed immediately. Sufficient acetate buffer (pH 4·6) was added to make a 1 in 1,000 w/v dilution and 0·02 ml. papain solution (50 mg./ml.) was added for each 10 ml. volume. The mixture was homogenized manually in a small tissue grinder, incubated in a water bath at 50° C. for one hour, steamed for 20 minutes and centrifuged. The clear supernatant was stored at − 20° C. until assayed. This 1 in 1,000 dilution was in most instances satisfactory for folic acid assay. Further dilution with distilled water was required to give a suitable solution for vitamin B_{12} assay.

Tissue blocks.

Approximately 1 gm. samples were blotted with filter paper, weighed and homogenized in 5 ml. acetate buffer (pH 4·6) for two minutes in a M.S.E. homogenizer. One ml. papain solution (50 mg./ml.) was added and the volume made up to 20 ml. with buffer. After incubation at 50° C. for one hour, the mixture was steamed and centrifuged as described for the needle biopsy specimens.

The papain solution (Difco) had no demonstrable vitamin B_{12} or folic acid activity.

In experiments designed to test the need for papain digestion, duplicate tissue blocks were processed in acetate buffer (pH 6·6) without incubation with papain.

Assays.

Vitamin B_{12} was assayed using either the Z strain of *Euglena gracilis* (Hutner, Bach and Ross, 1956) or *Ochromonas malhamensis* (Ford, 1953) as test organism. The extraction of the tissue for Ochromonas assay was carried out in the presence of cyanide and the assay medium prior to autoclaving contained 0·005 p.c. KCN. The Ochromonas cultures were grown without shaking over "warm-white" fluorescent tubes.

Folic acid activity was measured with a mutant strain of *Streptococcus lactis* R. (A.T.C.C. 8043). Growth was determined with a nephelometer. *Streptococcus lactis* is not specific for pteroylglutamic acid and the term "folic acid", as used in this paper, indicates material that will stimulate the growth of this organism under the conditions of the microbiological assay.

RESULTS.

Vitamin B_{12} concentrations of normal liver tissue blocks. Blocks of tissue removed from the right lobes of the livers of ten previously healthy subjects who had died suddenly were assayed by three techniques. The results are shown in Table 1. Euglena assay was performed on supernatants obtained both with and without prior digestion with papain. Papain digestion increased the yield from a mean value of 0·65 μg./gm. to a mean of 0·78 μg./gm. In nine of the

TABLE 1.

Vitamin B_{12} concentrations of normal liver tissue blocks.
(μg./gm. wet tissue)

No.	Sex and age	Euglena without papain digestion	Euglena with papain digestion	Ochromonas with papain digestion
1	M/64	0·51	0·63	0·68
2	M/20	0·79	0·90	0·99
3	F/16	0·74	0·62	0·51
4	M/68	0·45	0·67	0·63
5	M/75	0·78	0·86	1·03
6	M/74	0·53	0·61	0·49
7	F/83	0·58	0·88	0·69
8	F/68	0·67	1·02	0·74
9	M/50	0·80	0·85	0·78
10	M/83	0·64	0·80	0·71

ten samples, papain digestion gave the higher value. The mean value obtained by Ochromonas assay of papain-treated material was 0·73 μg./gm. In seven of the ten samples Euglena assay gave a higher value than Ochromonas assay.

Subsequent B_{12} assays were carried out on material extracted after digestion with papain and using *Euglena gracilis* as the test organism. With this method, the range of values in the ten normal liver samples was from 0·61 μg./gm. to 1·02 μg./gm.

Folic acid activity of normal liver tissue blocks. Table 2 shows that papain digestion markedly increased the folic acid content of the supernatants. The mean value for digested samples was 1·25 μg./gm. compared with that of 0·49 μg./gm. for material extracted without papain treatment. Subsequent folic acid assays were carried out on papain-treated material. With this method, the range of values in the ten normal liver samples was from 0·69 μg./gm. to 1·60 μg./gm.

TABLE 2.

Folic acid activity of normal liver tissue blocks.
(μg./gm. wet tissue)

No.	Without papain digestion	With papain digestion
1	0·78	1·50
2	0·21	1·10
3	0·37	1·20
4	0·54	0·83
5	0·30	1·60
6	0·55	1·50
7	0·86	0·69
8	0·38	1·20
9	0·37	1·60
10	0·58	1·30

Correlation between B_{12} and folic acid concentrations in normal liver samples. The vitamin B_{12} and folic acid concentrations shown in Tables 1 and 2 were tested for correlation by plotting on graph paper. No evidence of correlation of values from individual liver samples was found.

Vitamin B_{12} concentrations of needle biopsy specimens and tissue blocks from the same livers. Tissue blocks were obtained from both right and left lobes and a needle biopsy from the right lobe of livers of 12 hospital patients who came to autopsy. The vitamin B_{12} concentrations are shown in Table 3. Assuming that vitamin B_{12} is uniformly distributed in the liver, a comparison of the values obtained from right and left lobe tissue blocks should indicate the accuracy that can be expected from the method of B_{12} assay. It can be seen that the two sets of values are in good agreement. In no instance did either value differ from the mean by more than 10 p.c.

TABLE 3.

Vitamin B_{12} concentrations ($\mu g./gm.$) of liver blocks and needle biopsy specimens.

No.	Right lobe block	Left lobe block	Needle biopsy	Weight of needle biopsy (mg.)	Needle biopsy as percentage of mean tissue block
11	0·45	0·50	0·43	13·2	91
12	0·53	0·51	0·49	11·0	94
13	0·73	0·66	0·68	12·3	97
14	0·44	0·45	0·44	16·0	100
15	1·1	1·1	1·0	23·4	91
16	0·96	0·81	0·81	16·2	92
17	1·1	1·3	1·2	17·2	100
18	0·55	0·56	0·53	19·7	95
19	0·66	0·63	0·58	12·8	90
20	0·80	0·88	0·70	23·8	83
21	0·44	0·44	0·55	16·5	120
22	0·25	0·26	0·24	9·6	94

In this Table the needle biopsy values are recorded as a percentage of the mean tissue block values. In all samples, the needle biopsy value was within the range of 80 to 120 p.c. of the mean tissue block value. Weights of the needle biopsy samples varied from 9·6 to 23·8 mg., with a mean value of 16·0 mg.

Folic acid activity of needle biopsy specimens and tissue blocks from the same livers. These results are shown in Table 4. The two sets of values for right and left lobe blocks are in satisfactory agreement. In only one instance (No. 20) did either value differ from the mean by more than 10 p.c., and here the figure was 12 p.c. The needle biopsy value was within the range 80 to 120 p.c. of the mean tissue block value in nine of the 12 samples. The very low needle biopsy value obtained with sample No. 15 appeared to be due to a technical error.

TABLE 4.

Folic acid activity (μg./gm.) of liver blocks and needle biopsy specimens.

No.	Right lobe block	Left lobe block	Needle biopsy	Needle biopsy as percentage of mean tissue block
11	1·5	1·7	1·5	94
12	0·66	0·62	0·75	117
13	0·83	0·80	0·85	105
14	0·29	0·28	0·29	100
15	2·0	2·0	0·76	38
16	1·6	1·3	1·4	97
17	1·3	1·4	1·5	111
18	0·28	0·29	0·40	140
19	0·99	1·0	1·0	100
20	1·2	0·94	1·0	93
21	0·74	0·82	0·97	124
22	0·51	0·52	0·54	105

Assays of duplicate needle biopsy specimens of differing weights. Duplicate needle biopsy specimens were taken from liver samples Nos. 11 to 15. One of each pair was deliberately made small so that it would be technically difficult to process. The weights of the "satisfactory" biopsies ranged from 11·0 to 23·4 mg. and of the small biopsies from 2·5 to 8·6 mg. The B_{12} and folic acid results are shown in Table 5. In cases 11 and 15 where the small biopsy weights were 7·0 and 8·6 mg. respectively, there was good correlation between the values for "satisfactory" and small biopsies. In cases 12 and 14 (small biopsy weights 4·4 and 4·3 mg. respectively), the B_{12} results showed a marked discrepancy, while in case 13 (small biopsy weight 2·5 mg.), the folic acid results showed poor correlation.

TABLE 5.

Assay values of two needle biopsy samples of differing weights from the same liver specimens.

No.	Weight of biopsy (mg.)	B_{12} (μg./gm.)	Folic acid (μg./gm.)
11	13·2	0·43	1·5
	7·0	0·40	1·6
12	11·0	0·49	0·75
	4·4	0·21	0·80
13	12·3	0·68	0·85
	2·5	0·57	1·5
14	16·0	0·44	0·29
	4·3	0·71	0·29
15	23·4	1·0	0·76
	8·6	0·95	0·69

DISCUSSION.

The results indicate that satisfactory values for vitamin B_{12} and folic acid concentrations can be obtained from liver tissue blocks weighing 1 gm. or more. Correlation was good between right and left lobe samples, indicating that the assay techniques are reproducible and that these vitamins are uniformly distributed in the human liver. Surgical biopsy or autopsy samples are suitable for assay and most of the values reported in the literature have been derived from this type of material. Our mean value of 0·78 μg./gm. for the vitamin B_{12} content of normal liver blocks agrees closely with the values obtained by Swendseid, Hvolboll, Schick and Halsted (1957), using *L. leichmannii* as test organism. Our results confirm the findings of Ross and Mollin (1957) that papain digestion gives a higher B_{12} yield and that Ochromonas and Euglena methods produce comparable results with human liver.

The folic acid concentrations of normal human livers shown in Table 2 are considerably lower than those obtained by Girdwood (1953). The discrepancy is probably due to differences in the extraction procedure as assay values are influenced by the nature of the enzyme used for digestion (Girdwood, 1960) and the extraction time. It appears that higher values are obtained with an extract of chicken pancreas than with papain but these values are not necessarily the more accurate since the assay with *Streptococcus lactis* R. is not specific for pteroylglutamic acid. Papain was chosen for these studies as the small amount of needle biopsy material made it necessary to use one extract for both B_{12} and folic acid assays. Other workers have shown that papain extraction is suitable for liver B_{12} assay; the high B_{12} content of chicken pancreas would make an extract of this material unsuitable.

In view of the clinical evidence of interrelationship between vitamin B_{12} and folic acid, it was of interest that no correlation could be demonstrated between the concentrations of these substances in normal livers.

The results of the needle biopsy specimens shown in Tables 3, 4 and 5 suggest that reasonably accurate values can be obtained provided that the specimen weighs at least 7 mg. Apart from the folic acid result in case No. 15, which was grossly misleading, information of clinical value could have been obtained from all the needle biopsy specimens.

Cases 11 and 22 suffered from hepatic cirrhosis and the results from the biopsy specimens correlated well with the results from the tissue blocks in both instances.

Acknowledgments. Miss M. C. Dawbarn, C.S.I.R.O., Adelaide, kindly supplied us with a culture of *Ochromonas malhamensis*. Mr. R. E. Davis was responsible for the folic acid sensitive mutant strain of *Streptococcus lactis* R.

REFERENCES.

Drouet, P. L., Wolff, R., Karlin-Weissman, R., and Rauber, G. (1951): Bull. Soc. med. Hôp. Paris, 67, p. 281.

Ford, J. E. (1953): Brit. J. Nutrit., 7, p. 299.

Girdwood, R. H. (1953): Blood, 8, p. 469.
Girdwood, R. H. (1960): In "Advances in Clinical Chemistry". Eds. H. Sobotka and C. P. Stewart (Academic Press, New York and London), p. 273.
Hutner, S. H., Bach, M. K., and Ross, G. I. M. (1956): J. Protozool., 3, p. 101.
Jhala, C. I., and Gadgil, R. K. (1960): J.J.J. Group Hosp. and Grant Med. Coll., 5, p. 36.
Nelson, R. S., and Doctor, V. M. (1958): Ann. int. Med., 49, p. 1361.
Ross, G. I. M., and Mollin, D. L. (1957): In "Vitamin B_{12} und Intrinsic Factor" Ed. H. C. Heinrich (Enke, Stuttgart), p. 437.
Swendseid, M. E., Hvolboll, E., Schick, G., and Halsted, J. A. (1957): Blood, 12, p. 24.

GENETIC STUDIES ON LYSOGENY IN *PSEUDOMONAS AERUGINOSA*[1]

by J. B. EGAN[2] AND B. W. HOLLOWAY

(Department of Bacteriology, University of Melbourne, Parkville, Victoria).

(*Accepted for publication 16th November, 1960.*)

SUMMARY.

Mutants of the *Pseudomonas aeruginosa* temperate phage D3 have been isolated which are characterised by a clear plaque and a reduced frequency of lysogenisation. They have been classified into three groups on their ability to co-operate in mixed infection to give a lysogenisation frequency characteristic of the wild type. Genetic recombination has been demonstrated for phage D3 and a preliminary linkage map constructed. Crosses between representatives of the three groups of clear plaque mutants confirm the genetic diversity indicated by the co-operation test.

INTRODUCTION.

Mutants characterised by a clear plaque morphology and a reduced frequency of lysogenisation have been isolated from a variety of temperate phages. Kaiser (1957) has shown that the clear plaque mutants of the coliphage lambda can be divided into three groups, each group possessing a particular frequency of lysogenisation lower than the wild type. However, after mixed infection with representatives of any two groups the frequency of lysogenisation is that of the wild type. This reaction has been termed co-operation and involves the presence of two complementary genotypes within the doubly infected bacterium to give the full phage genome necessary to establish lysogeny. This takes place in the absence of genetic recombination between the two deficient genomes. By means of linkage studies, Kaiser showed that the loci of clear plaque mutants are restricted to one region of the chromosomes which he called the c-segment. This segment can, in turn, be divided into three discrete sub-regions which correspond to the groups established by co-operation. Levine (1957) has described a similar situation with *Salmonella typhimurium* and phage P22.

An investigation of clear plaque mutants in phage D3 was initiated to obtain a better understanding of lysogeny in *Pseudomonas* phages and to establish a basis of genetic techniques for this group of phages.

---

[1] This work was supported by a grant from the National Health and Medical Research Council, Canberra.

[2] Present address: Department of Biophysics, University of Colorado Medical Centre, Denver, Colorado.

MATERIALS AND METHODS.

Media. Except where stated in the text, liquid and solid media were the same as those used by Holloway *et al.* (1960). Indicator Agar (IA) (for recombination experiments): tryptone (Difco) 10·0 gm., yeast extract (Difco) 5·0 gm., glucose 8·5 gm., sodium chloride 0·5 gm., agar 15·0 gm., distilled water 1 l. The glucose was sterilised and added separately.

Bacterial strains. Pseudomonas aeruginosa strain 1 (Holloway, 1955) was used as the indicator strain. The derivatives 1 (D3) lysogenic for wild type D3 and 1 D3-r, resistant to D3, were obtained by the usual techniques.

Bacteriophage. The temperate wild type phage D3 plated on strain 1 formed solid centre circular plaques 2-3 mm. in diameter surrounded by a circular halo with an overall diameter of up to 8 mm. (Holloway *et al.*, 1960), and clear plaque mutants occurred in stocks at a frequency of 2×10^{-3}. The D3 prophage is inducible by ultraviolet irradiation. Plaque morphology mutants were isolated by the method of Weigle (1953) and will be described below. General phage techniques are the same as those described by Adams (1959). Bacterial strain 1 has a generation time of 30 minutes, phage D3 has a latent period of 63 minutes and a burst size of approximately 100. No co-factor for adsorption of D3 was detected and under the experimental conditions used up to 95 p.c. adsorption was obtained.

Measurement of the frequency of lysogenisation. Bacteria at a known density of about 5×10^7 cells/ml. in the logarithmic phase of growth were infected with phage at a multiplicity of 10. Infection was allowed to proceed for 60 minutes then, shortly before lysis, the culture was diluted into antiphage serum diluted in NYB to neutralise free phage. After a further 15 minutes the infection mixture was diluted further and assayed for bacterial survivors. The colonies formed in this assay were thus derived either from lysogenic or uninfected cells. By replica plating on to a lawn of bacterial cells (strain 1) the colonies derived from lysogenic infections could be distinguished from the non-lysogenic. For each experiment of this type, the actual multiplicity of infection was experimentally determined.

Phage recombination. A culture of 5×10^7 bacteria/ml. in the logarithmic phase of growth was infected at a multiplicity of infection of 10 for each parental phage and after 15 minutes' adsorption at 37° C. was diluted tenfold into antiphage serum diluted in broth so as to give 99 p.c. neutralisation in 15 minutes. After a further 15 minutes at 37° C. the infected culture was diluted 1/1000 in prewarmed Nutrient Yeast Broth (NYB) and 10 ml. of this dilution were incubated with aeration for 90 minutes at 37° C. The phage liberated from the lysis of the infected bacteria was plated at suitable dilution on indicator agar for the scoring of the various plaque types.

RESULTS.

Fifty-four clear plaques were isolated and stocks prepared from each using the layer-agar method (Adams, 1959). The stocks were stored over chloroform at 4° C. and were quite stable. When a loopful of each of these stocks was placed on the surface of a lawn of 1 each isolate gave a clear area of lysis. This lytic reaction did not occur when 1 (D3) was used for the lawn. These facts identify the mutants as c-type mutants analogous to those in lambda (Kaiser, 1957) and P22 (Levine, 1957). The existence of different types amongst these clear plaque mutants was shown in several ways.

Co-operation. Each clear plaque mutant, together with bacteria of strain 1, was in turn used to seed a layer agar. Immediately the agar solidified, loopfuls

from the remaining isolates were spotted individually and plates incubated overnight at 37° C. Confluent lysis of the lawn occurred over the whole plate except in certain cases. This occurred in those regions where loopfuls of certain clear plaque mutants had been spotted in the surface of the lawn and there bacterial growth was found in the area spotted. It was concluded that this was due to the phenomenon of co-operation resulting in bacterial survivors lysogenic for either or both of the clear plaque mutants used in the infection. Controls of each mutant on a bacterial lawn and on the surface of a meat infusion agar (MIA) plate confirmed the clear plaque characteristics and the bacterial sterility of each phage preparation. Optimal concentrations of bacteria and phage were determined empirically by varying the concentrations of the spotted and the layered phage and it was found that seeding the layer with $1\text{-}2 \times 10^8$ bacteria and 5×10^7 phage particles, then adding loopfuls (2 mm. loop) from stock preparations of 5×10^{10} phage particles per ml. gave the best results. In all, 1080 of the 2916 possible combinations were tested and the typical response of each group in a screening test is shown in Fig. 1. The overall results indicated that the 54 c-mutants isolated could all be placed into three groups denoted c1, c2 and c3.

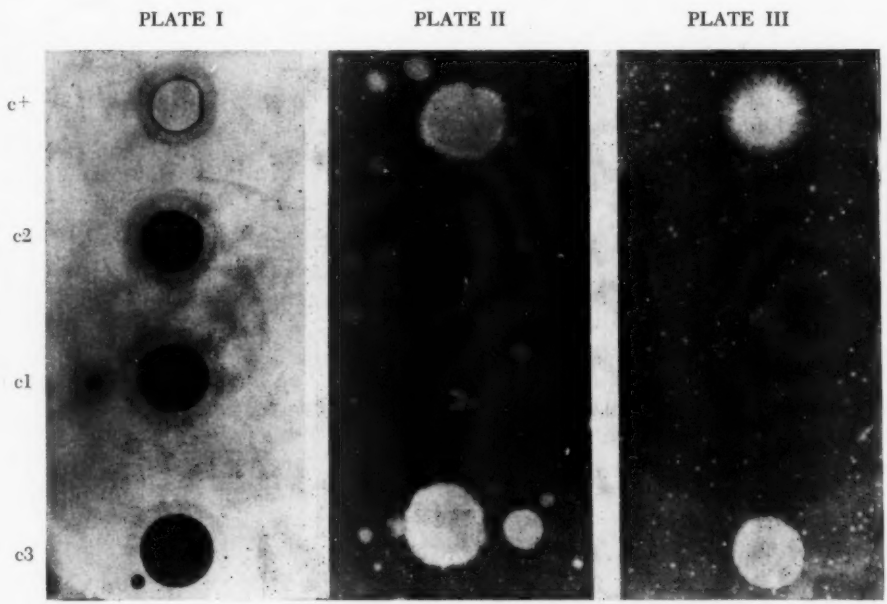


Fig. 1. Wild type (c+) and clear plaque mutants c1, c2 and c3 of *Ps. aeruginosa* phage D spotted on a lawn of bacterial strain 1 (plate I), a lawn of strain 1 + c1 phage (plate II) and strain 1 + c2 phage (plate III). The phages spotted are in order from the top c+, c2, c1, c3.

It is seen that the wild type phage D3^{c+} gave a solid centre spot on the lawn of sensitive bacteria (plate I) and bacterial survival on lawns of strain 1 + c1 phage (plate II) and strain 1 + c2 phage (plate III). c1 phage gave a clear spot on plate I and no bacterial survival on plates II and III. This type of response will be referred to as: c1 did not co-operate with c1 or c2. 49 of the 54 mutants tested belong to this group. c2 phage gave a clear spot on plate I, bacterial survival on plate II and no bacterial survival on plate III, i.e. c2 co-operates with c1 but not with c2. Two of the 54 mutants tested belong to this group. c3 phage gave a clear spot covered with minute colonies on plate I, and bacterial survival on plates II and III, i.e. c3 co-operates with c1 and c2. Three of the 54 mutants belong to this group.

It is seen that the co-operation between c1 and c2 is not truly reciprocal under the conditions used. When c2 is the co-operation partner in the lawn and c1 is added by means of a loop no co-operation results. The reverse process when c1 is in the lawn and c2 is added subsequently results in co-operation. Experiments to see if some critical ratio of c1 and c2 multiplicities was necessary for co-operation to occur, did not yield any useful information. One possible explanation is that for co-operation to occur infection with c1 must precede infection with c2. A similar situation has been described by Kaiser (1957) for lambda.

The three groups of c-mutants have characteristic back mutation rates to the wild type condition. The c1 group has not been observed to back mutate at all, c2 back mutates at a frequency to give one wild type plaque in about 5,000 clear plaques while c3 has a much higher rate of back mutation, three wild type plaques occurring to every 100 mutant plaques. The back mutation of c3 to c^+ is not responsible for the bacterial survival observed in co-operation experiments with c3. The bacteria isolated from the growth resulting when a loopful of c3 is placed on a c1 lawn were predominantly lysogenic for the clear plaque mutant, not the wild type c^+.

No demonstration of co-operation could be made with c3 as the seeded phage in the lawn, due to the type of lawn formed by the c3 mutant. Fortunately the non-reciprocal nature of the c1-c2 interaction dispenses with the need for a third type for differentiation of the groups. One representative isolate was chosen from each of the three groups for all subsequent experiments.

Plaque appearance. From Fig. 1, plate I, it can be seen that the wild type c^+, when spotted first on a lawn of strain 1, gives a solid centre of lysogenic bacteria surrounded by an area of lysis and then a halo of reduced growth. On the other hand, c1 has a clear centre with a few scattered phage resistant colonies bordered by a thin turbid ring and surrounded by the halo. The c2 spot, however, has a centre which is very lightly turbid compared with c1, a turbid border and the whole surrounded by the halo. A few lysogenic cells could be isolated. The c3 spot has a clear centre covered with minute colonies of lysogenic cells, a solid edge of bacterial growth, a lytic ring, and the halo outside.

Genetic recombination. Differentiation of the three groups of c-mutants can also be made by recombination experiments. These will be discussed in the appropriate section below.

The c-mutant established as prophage through co-operation.

Bacteria from the survival spots of c2 and c3 on c1 (Fig. 1, plate II) and colonies isolated from the otherwise completely lysed regions of c1, c2 and c3 spotted on strain 1 (Fig. 1, plate I) were purified by passage through anti-phage serum broth and subsequent single colony isolation. Twelve colonies from each were selected and inoculated into NYB. After overnight incubation at 37° C. each culture was subcultured into 3 ml. of an actively growing culture of strain 1 to propagate the phage. After a period of three hours the cultures were centrifuged and chloroform added to the supernatants. Loopfuls of each of these were spotted on to strain 1 to determine if phage was present, and if so, of what plaque and c-type. The lysogenic nature of the original colonies tested in this way was confirmed by their induction by UVL to produce phage. The results are shown in Table 1.

TABLE 1.

The c-type of the phage released from bacterial survivors due to co-operation.

Bacterial survivors isolated after	No. of colonies	No. of colonies releasing c-mutant phage	No. showing co-operation with c1 in lawn
1. c1 infection	12	0	0
2. c2 infection	12	6	3
3. c3 infection	2	2	2
4. c1 + c2 infection	11	11	8
5. c1 + c3 infection	10	7(a)	7

(a) The remaining 3 released c^+ phage.

It is quite clear that in mixed infection with c1, both c2 and c3 gave bacterial survivors that were lysogenic for c2 and c3 respectively. As indicated by the reactions of Fig. 1 there is unfortunately no conclusive test for the liberation of c1 under these conditions.

Lysogenisation frequencies of c-mutants.

Attempts to adapt the triple-layer technique (Kaiser, 1957) to determine lysogenisation frequencies of the *Ps. aeruginosa*—D3 system were unsuccessful. An alternative method involving replica plating was, however, used with some success. Such a method proved satisfactory for infections with c^+ or c3 but technical difficulties arose with c1 and c2 infections and the method is not sufficiently accurate to provide quantitative evidence in support of co-operation. With this method it was found that the lysogenisation frequency of c^+ infections was between $2{\cdot}3\text{-}4{\cdot}8 \times 10^{-1}$ and that for c3 was between $1{\cdot}7\text{-}2{\cdot}7 \times 10^{-1}$.

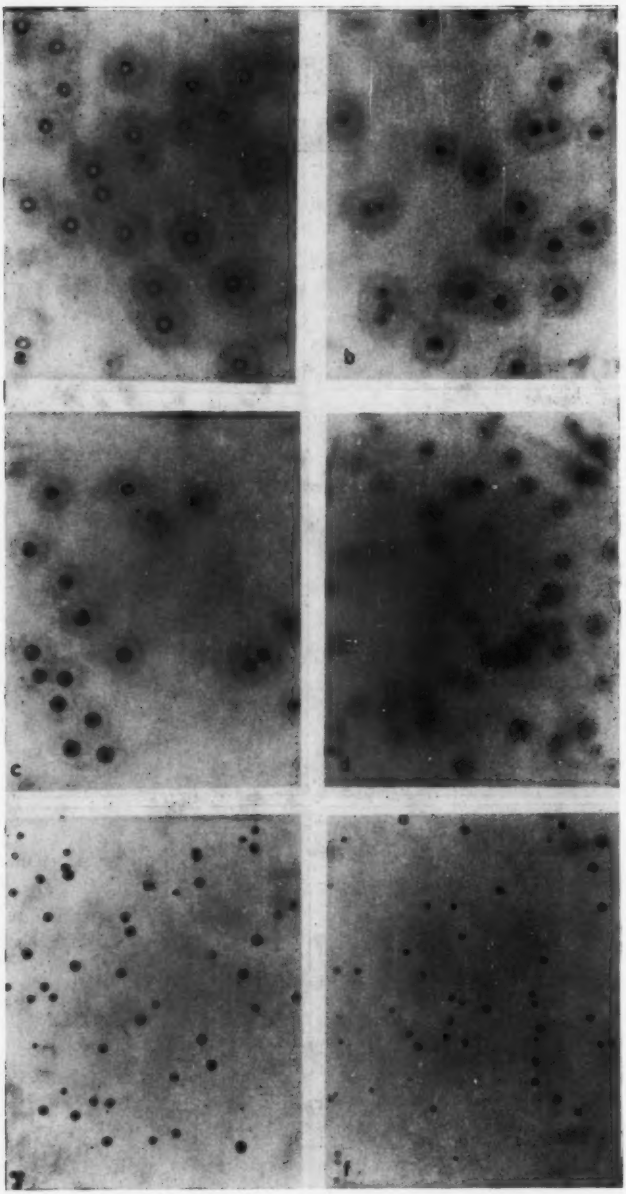


Fig. 2. Plaque morphology of mutants and recombinants of phage D on indicator *Ps. aeruginosa* strain 1. (a) wild type; (b) clear plaque type c1; (c) grand clear (c1 g); (d) minute clear (c1 m); (e) haloless clear (c1 *l*); (f) haloless solid centre (c+ *l*).

Genetic recombination in phage D.

Recombination studies on the c-mutants of lambda by Kaiser and of P22 by Levine indicated that the loci of these mutants occupied a discrete segment of the phage chromosome which extended over a distance of only 1-2 recombination units. Moreover, Kaiser located the mutants of each co-operation group within a distinct region of this c-segment.

In order to demonstrate recombination in phage D3 a number of plaque morphology mutants were obtained by the method of Weigle (1953). A c1 mutant preparation was irradiated to a survival of 5×10^{-5} measured as plaque-forming particles on non-irradiated bacteria. This irradiated phage was plated on bacteria irradiated to a survival of 2×10^{-1} using indicator agar as it was found that this medium gave better plaque differentiation than that used for ordinary assays. Three mutants were isolated (see Fig. 2) and their properties may be compared with the parental type as follows:

Parental c1: a clear centre surrounded by a band of faint turbidity, this having an entire outer edge, the whole surrounded by a halo.

Mutants c1 *l*: *haloless*: A clear centre, irregular outer edge and lacking the halo of the wild type.

c1 g: *grand*: large clear centre, smooth outer edge with a halo.

c1 m: *minute*: a pin-point clear centre, with same general appearance of the parental c1 type but smaller.

Three host range mutants were isolated with the following characteristics. These have not been used for recombination experiments to date.

c1 h 97: plates on *Ps. aeruginosa* strain 97, this strain being resistant to the wild type D3.

c1 h D-r: plates on 1 D-r.

c1 h 97 v: a virulent mutant capable of overcoming prophage immunity indicated by its plating on 1(D). Derived from c1 h 97.

No back mutation was observed with any of these mutants.

With the appropriate stocks derived from these mutants and using the techniques previously described, a series of two and three factor crosses were made. The results are given in Table 2.

From these data the most probable linear sequence which can be deduced is as follows:

$$m - c2 - c1 - c3 - g - l.$$

It is seen that the three c-loci are not separated by any of the other loci tested. c1 and c2 are very closely linked although the data do not indicate any close linkage of c3 with c1 or c2. The production of wild type recombinants in crosses of c1 $\times$ c3, c2 $\times$ c3 and c1 $\times$ c2 confirms the differentiation of these types

TABLE 2.

Genetic recombination in phage D3.

Cross No.	Parents	Progeny		p.c. age recombination
		Parental types	Recombinant types	
1	c1 l × c1$^+$ l^+	1964 c1 l, 1166 c1$^+$ l^+	431 c1 l^+, 231 c1$^+$ l	17·5
2	c2 l × c2$^+$ l^+	777 c2 l, 1393 c2$^+$ l^+	155 c2l^+, 433 c2$^+$ l	21·3
3	c3 l^+ × c3$^+$ l	421 c3 l^+, 729 c3$^+$ l	123 c3l, 21 c3$^+l^+$	11·1[a]
4	m$^+$c1g × mc1g$^+$	150 m$^+$c1g, 581 mc1g$^+$	93 m$^+$c1g$^+$, 101 mc1g	21·4
5	c1g$^+$ l × c1gl^+	648 c1g^+l, 291 c1gl^+	0 c1g$^+l^+$, 0 c1g l	0
6	mc1 l^+ × m$^+$c1 l	706 mc1 l^+, 410 m$^+$c1 l	207 m$^+$c1 l^+, 176 mc1 l	25·6
7	c2c1$^+$ × c2$^+$c1	4000(c2c1$^+$, +c2$^+$c1 + c2c1)[b]	4 c2$^+$c1$^+$	0·2
8	c1c3$^+$ × c1$^+$c3	2951(c1c3$^+$, +c1$^+$c3 + c1c3)[b]	307 c1$^+$c3$^+$	12·4[a]
9	c2c3$^+$ × c2$^+$c3	1478(c2c3$^+$, +c2$^+$c3 + c2c3)[b]	203 c2$^+$c3$^+$	17·8[a]
10	mc1 l^+ × m$^+$c1^+l	679 mc1 l^+ 416 m$^+$c1^+l	15 m$^+$c1 l^+, 23mc1^+l 178mc1 l, 156 m$^+$c1$^+l^+$ 20 m$^+$c1 l, 11mc1$^+l^+$	

[a] recombination percentage corrected for back mutation of c3 to c$^+$

[b] recombinant not distinguishable from parental plaque type.

on the grounds of their lytic and co-operation reactions. It is curious that haloless (*l*) and grand (g) should show such close linkage. The unequal recovery of each parental type in certain crosses may indicate some degree of selection.

DISCUSSION.

The existence of three groups of clear plaque mutants of phage D is clearly indicated not only on their reactions in the co-operation phenomenon and their phenotypic appearance, but also from the genetic recombination experiments and together these results confirm those obtained in lambda and P22. The difficulty of obtaining quantitative data of lysogenisation frequencies has limited further work on the nature of co-operation in phage D3.

Two other features of this work will merit further investigation. Firstly the recombination frequencies obtained and the availability of mutants make studies of recombination highly feasible. In this respect recent results with phages T1 and P22 (Trautner and Bresch, 1960) indicate that, with increasing multiplicities of infection, recombination frequencies also increase. As the crosses described in this paper have been done with multiplicities between 5 and 10 the relatively high recombination frequencies observed may be more apparent than real. This effect may be the explanation of the apparent enlarged distances between c-type mutants c1 (or c2) and c3, as compared with lambda and P22.

The second feature for future consideration concerns the genetic stability of the c region. The ease with which c-mutants are isolated and the frequency of back mutation of one of the types (c3) indicate how unstable this region is and as such it would provide a suitable experimental system for a study of induced mutation in phage. This type of experiment has recently been described by Kaplan *et al.* (1960) using a *Serratia* phage.

Acknowledgments. One of us (J.B.E.) was supported by a Commonwealth Postgraduate Scholarship while on leave from the Commonwealth Serum Laboratories.

REFERENCES.

Adams, M. H. (1959): "Bacteriophages". Interscience Publishers, New York.
Bertani, L. E. (1958): Abstracts. VIIth International Congress for Microbiology, Stockholm, 1958, p. 52.
Holloway, B. W. (1955): J. gen. Microbiol., 13, p. 572.
Holloway, B. W., Egan, J. B., and Monk, M. (1960): Austral. J. exp. Biol., 38, p. 321.
Kaiser, A. D. (1957): Virology, 3, p. 42.
Kaplan, R. W., Winkler, U., and Wolf-Ellmaur, H. (1960): Nature, 186, p. 330.
Levine, M. (1957): Virology, 3, p. 22.
Trautner, T. A., and Bresch, C. (1960): Quoted in Bresch, C. (1959). Ann. Rev. Microbiol., 13, p. 313.
Weigle, J. J. (1953): Proc. Nat. Acad. Sci. (Wash.), 39, p. 628.

A DEGENERATION STUDY OF THE SOMAESTHETIC AFFERENT SYSTEMS IN THE MARSUPIAL PHALANGER, *TRICHOSURUS VULPECULA*

by J. K. A. CLEZY, BARBARA J. DENNIS AND D. I. B. KERR

(From the Department of Human Physiology and Pharmacology, University of Adelaide).

(*Accepted for publication 22nd December, 1960.*)

SUMMARY.

Degenerating fibres in the medial lemniscus and anterolateral columns of the phalanger have been traced using the Marchi technique. The medial lemniscus, arising from a lesion in the dorsal column nuclei, decussates in the lower medulla and follows a contralateral pathway, except in its most caudal extent where some degeneration on the ipsilateral side at inferior olivary levels is suggestive of a small component of uncrossed fibres. This ipsilateral representation soon fading, the pathway thereafter is completely crossed. It remains in a ventromedial situation throughout the midbrain, and penetrates the subthalamus to terminate in n. ventralis posterolateralis of the thalamus.

Anterolateral column lesions in the cervical cord result in ascending degeneration which can be traced into the medulla as an ipsilateral compact anterolateral marginal band (Gower's tract), together with a scatter of degeneration medially into the reticular formation up to the level of the superior olives. At upper pontine levels, fibres of the ventral spinocerebellar tract arch dorsally and turn back to reach the cerebellum through the superior cerebellar peduncle. No myelinated spinotectal fibres are to be found, and there is no direct passage of myelinated fibres to the thalamus.

INTRODUCTION.

The validity of the interpretation of the results of Marchi degeneration has often been queried (e.g. Bowsher *et al.*, 1960). However, provided the standard procedures are followed, the results are acceptable with the qualification that the terminal degeneration is not disclosed, because the osmic acid stains only the myelin sheath leaving the naked terminals untouched. The Marchi method effectively stains myelinated systems while with the silver methods it is more difficult to assess the fibre size and degree of myelination of degenerating fibres. In this study the Marchi technique has been preferred because it stains the larger myelinated fibres, from which we consider we have recorded evoked potentials in afferent pathways (Dennis and Kerr, 1961; Kerr, 1961), and forms a useful preliminary to experiments involving the more detailed silver techniques. Sufficient is known of the relationships of Marchi and silver degeneration studies

in other animals to indicate that Marchi degeneration discloses the approximate termination of the main afferent fibre tracts even though final termination may not be revealed.

METHODS.

Marchi degeneration studies were carried out in 10 marsupial phalangers where lesions were placed under aseptic conditions in the cervical cord, generally by hemisection at the level of C_1-C_2, and in the dorsal column nuclei. After 14-21 days the animals were again anaesthetized and the brains perfused through the aorta via the left ventricle using the magnesium sulphate-potassium bichromate solution of Swank and Davenport. Subsequently paraffin sections were cut from small blocks of brain which had been stained with osmic acid by the Swank and Davenport (1934) modification of the Marchi method for degenerating myelin.

RESULTS.

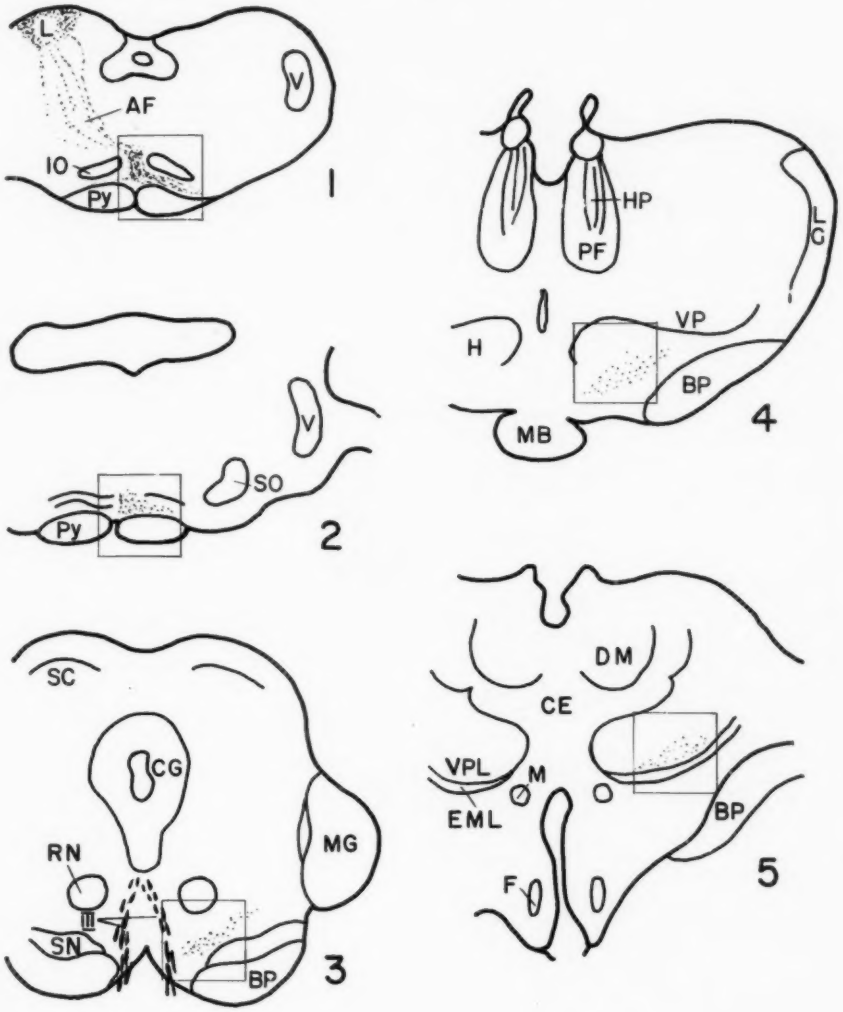
Medial lemniscus.

Following lesions to the nuclei of the dorsal columns of the phalanger, degeneration of the medial lemniscus could be traced to the thalamus. There is very little remarkable about the passage of the fibres in the medial lemniscus up to the tectal level. Decussation of the lemniscus is essentially completed by the level of the upper third of the inferior olive; the fibres, after crossing, come to lie over the pyramid spread as a band with its lateral extent reaching almost to the ventral brain surface at the lateral margin of the pyramid. Medially the fibres extend somewhat more dorsally giving the tract a comma shape in the medulla (Figs. 1, 2, 6, 7). A few fibres are found over the pyramid on the side of the lesion at inferior olivary levels, but crossing is soon completed.

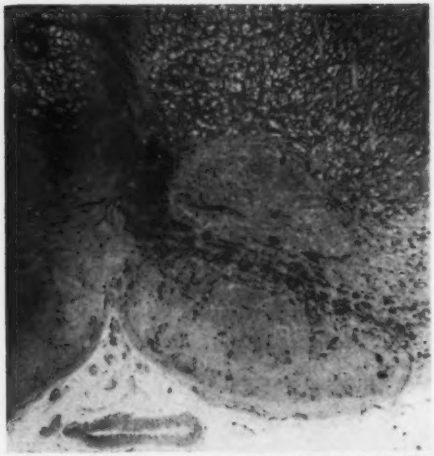
Higher in the brainstem at the collicular level the fibres remain bunched in a ventro-medial position adjacent to, and almost touching, the interpeduncular nucleus; they are thus not spread so far laterally as at comparable levels in the cat. This more medial and ventral course is pursued to the level of the extent of the IIIrd nerve, where the most medial degeneration is almost penetrated by the traversing IIIrd nerve (Figs. 3, 8). At this level the lemniscus of the phalanger is distinctly different in its course from that of higher forms in which dorsolateral migration of the tract rapidly occurs in the midbrain to a position immediately adjacent to the medial geniculate nucleus (Ranson and Ingram, 1932). This difference is partly due to the fact that the medial geniculate bodies extend more caudally in the phalanger. Even so, a little higher in the brain, where the thalamus begins and the mammillary bodies make their appearance on the ventral surface of the brain, the phalanger lemniscus is still found in a relatively ventro-medial situation (Figs. 4, 9), penetrating the subthalamus rather than the ventral posterior thalamus as happens in higher forms. At this level the Marchi granules are more dispersed and have begun to thin, indicating that the termination of the system is approaching.

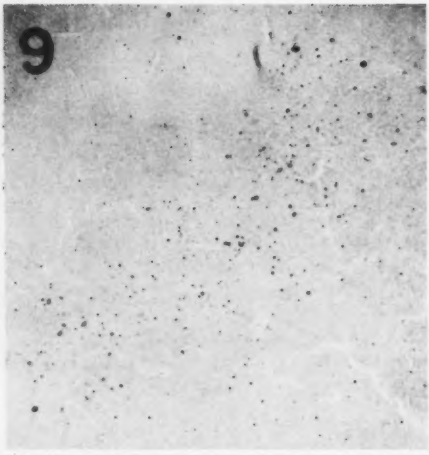
At the level of n. ventralis posterolateralis of the thalamus there is a rapid

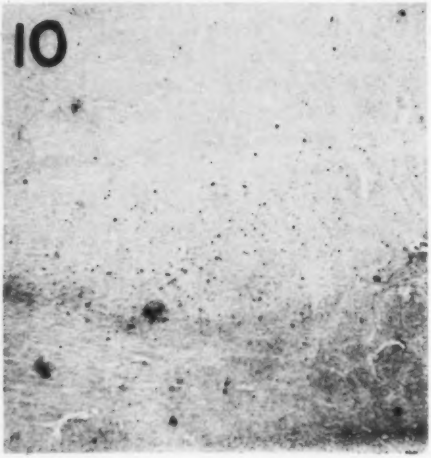
migration of the system through the external medullary lamina into the nucleus itself (Figs. 5, 10). The Marchi granules here become vanishingly fine, and termination is effected as a narrow band above the external lamina.

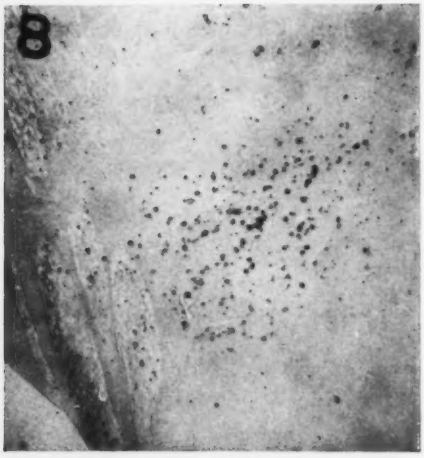


Figs. 1-5. Marchi degeneration (stippled) resulting from a lesion in the dorsal column nuclei on the left side. Phalanger. The squares enclose the areas corresponding to the photomicrographs in Figs. 6-10.

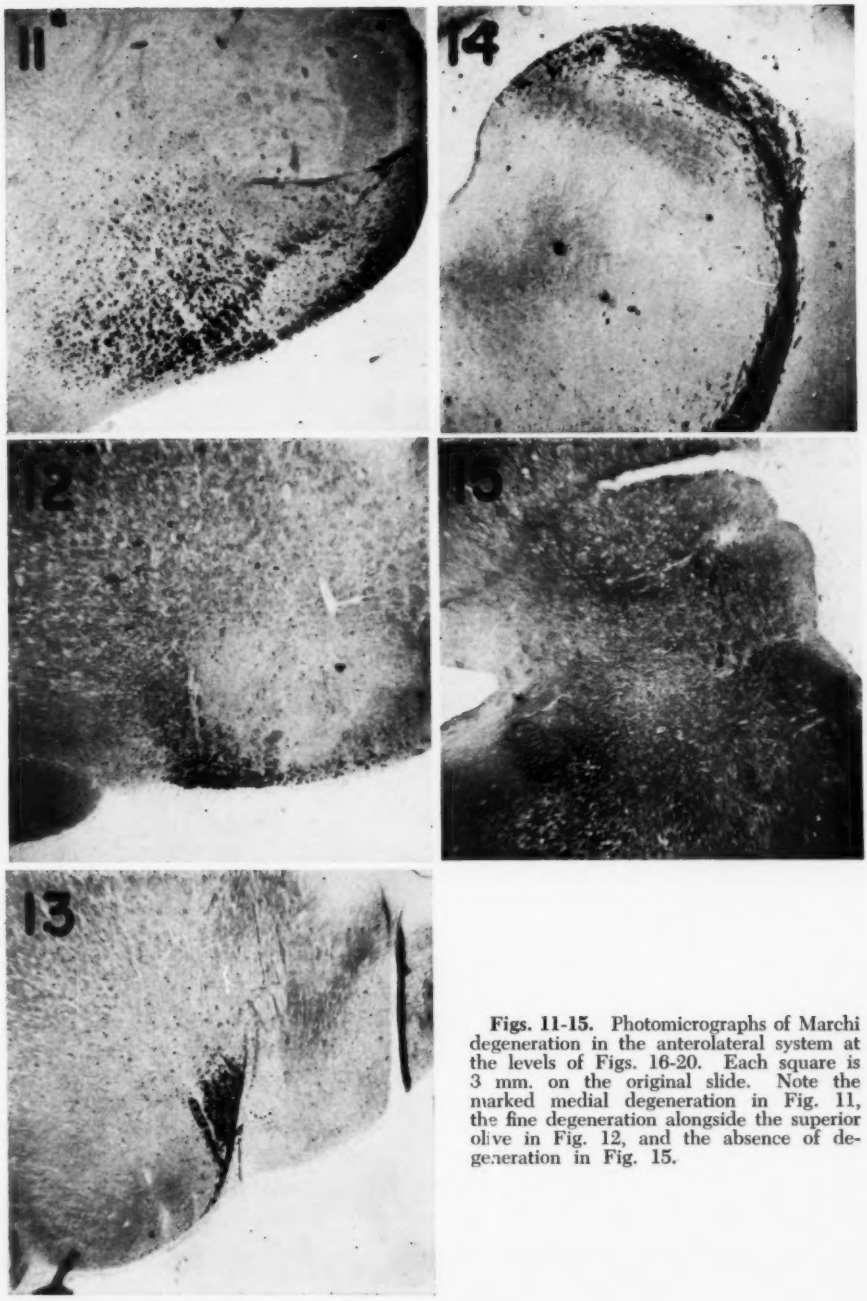






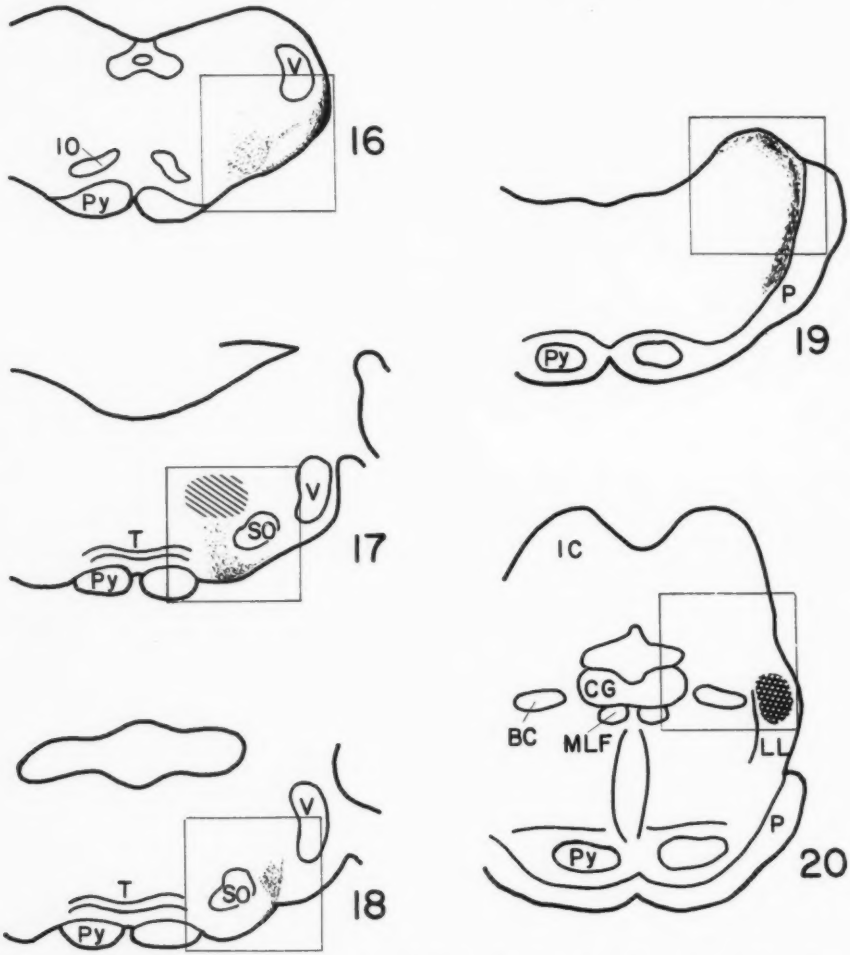


Figs. 6-10. Photomicrographs of Marchi degeneration at the levels of Figs. 1-5. Each square is 1·7 mm. on the original slide.



Figs. 11-15. Photomicrographs of Marchi degeneration in the anterolateral system at the levels of Figs. 16-20. Each square is 3 mm. on the original slide. Note the marked medial degeneration in Fig. 11, the fine degeneration alongside the superior olive in Fig. 12, and the absence of degeneration in Fig. 15.

Matzke (1951) has described an aberrant group of lemniscal fibres, probably originating from the dorsal column nuclei, in the cat. These fibres in the cat follow a course very similar to that of the medial lemniscus in the phalanger in that they penetrate the thalamus from below, through the subthalamus, rather



Figs. 16-20. Marchi degeneration (stippled) in the anterolateral system resulting from hemisection of the cord at C_1-C_2 on the right side. Phalanger. The squares enclose the areas corresponding to the photomicrographs in Figs. 11-15. The hatched area in Fig. 17 indicates the synaptic area of the central tegmental fasciculus. Evoked responses of the anterolateral system are recorded from the cross-hatched area in Fig. 20.

than entering the posterior thalamus adjacent to the medial geniculate nuclei as is more common in the higher forms.

In the phalanger the lemniscus covers only a small extent of the brainstem throughout its length, and it is relatively difficult to identify by neurophysiological techniques. Tactile evoked potentials referable to the medial lemniscus are, however, recorded from areas in the brainstem corresponding to the Marchi degeneration that results from lesions in the dorsal column nuclei, and the two methods of identification correlate satisfactorily.

The region of the thalamus indicated here as the termination of the medial lemniscus agrees well with that defined by retrograde degeneration following lesions of the phalanger sensory cortex (Goldby, 1943). Actually, these lesions lay in somatic area 1, as defined by evoked potentials studies (Adey and Kerr, 1954). There is thus every justification for calling this region (levels A11-A13 in Dennis and Kerr, 1961) nucleus ventralis posterolateralis of the phalanger thalamus, homologous with the termination of the medial lemniscus in the thalamus in higher forms. (Le Gros Clark, 1936; Ranson and Ingram, 1932; Ferraro and Barrera, 1936; Bowsher, 1958).

Anterolateral system.

Although Wallenberg (1900) was able to follow the spinothalamic system from the upper cervical cord up to the level of the medial geniculate nucleus in the rabbit, subsequent workers, with the exception of Geberetzoff (1939), have failed to demonstrate a spinothalamic tract by Marchi methods in animals below primates. This discrepancy is possibly due to the failure of the Marchi method because of the small diameter of the fibres involved (Häggquist, 1936). However, Getz (1952), Mehler (1957), Nauta and Kuypers (1958), and Anderson and Berry (1959) have traced a sparse system of spinothalamic fibres by silver degeneration methods in cats. In lower forms (Virginian opossum) even these small fibres are almost absent (Mehler, 1957). Notwithstanding this, in the Australian phalanger it is possible to record relatively large potentials, conveyed at a short latency, from the upper brainstem regions which are known to contain the spinothalamic tract in primates. However, no Marchi degeneration could be traced to these regions in the phalanger following hemisection of the cervical cord.

The anterolateral system, as seen with the Marchi method in the phalanger, is largely made up of ventral spinocerebellar fibres. In the lower medulla the degeneration arising from a cervical hemisection lies quite lateral, with the dorsal-spinocerebellar component, which is not considered in detail here, separating dorsally (Figs. 11, 16). The anterolateral system degeneration appears in two groups which join ventrally; a medial portion of more sparsely distributed fibres in the reticular formation, and a denser marginal band. The two groups are separated by a slim nucleus, the lateral reticular nucleus (Figs. 11, 16).

The marginal band of fibres lies just lateral to the pyramid and medial

lemniscus on the brain surface at the level of the facial nucleus and at the lower extent of the superior olives. At these levels the medial group of fibres is found as a dorsal extension of finer degeneration which extends into the reticular formation medial to the facial nucleus and superior olive (Figs. 12, 17). Ascending the brain, the fibres of the medial group become finer and appear separated from the main anterolateral projection as this migrates laterally beneath the superior olives, until finally the medial group is no longer discernible. At this stage the anterolateral projection lies lateral to the rostral pole of the superior olive (Figs. 13, 18).

The medial group of degenerating fibres seems destined for the synaptic area of the central tegmental fasciculus (Dennis and Kerr, 1961), but the Marchi method is of no use in establishing its ultimate termination, beyond the obvious thinning of degenerating granules as the termination is approached. It seems likely that this medial system goes to the origin of the central tegmental fasciculus; certainly it cannot be traced rostrally beyond it. It is thus a spino-reticular system rather than being spino-tectal as Poirier and Bertrand (1955) have suggested in higher forms. The presumed synaptic region is indicated by the hatched area in Fig. 17.

The anterolateral system migrates rapidly into a dorsal situation at the upper pons. The fibres arch rostrally and dorsally, and then turn back to enter the cerebellum in the superior cerebellar peduncle. The brain has been cut at the plane of this sweeping passage in Figs. 14, 19.

No fibres can be detected entering the colliculi (Figs. 15, 20). The usual situation for such fibres in higher forms is within the boundaries of the lateral lemniscus. The region from which limb afferent potentials could be recorded at this level (Dennis and Kerr, 1961) is indicated by the cross-hatched area in Fig. 20. The corresponding region in Fig. 15 is clear of any degeneration.

One would expect to find large myelinated fibres in the anterolateral system above the pontine level because conduction times of afferent evoked responses recorded at the thalamus are more consistent with transmission in a myelinated system than in finer non-myelinated fibres of the type which would not give a Marchi stain. A synaptic delay of 1·5 msec. would suffice to produce the observed latencies of evoked potentials in the anterolateral system which lags the myelinated lemniscus by this amount (Dennis and Kerr, 1961). It would seem that the afferent systems of spinal origin have not progressed to the extent of establishing direct large-myelinated fibre contact with either the thalamus or the tectum in the phalanger. We interpret the fact that no medullated fibres of the anterolateral system can be demonstrated degenerating rostral to pontine levels not as failure of the Marchi technique to reveal degeneration, for the ventral spinocerebellar system is well disclosed, but as evidence that the medullated fibres of the anterolateral system synapse within the medulla. The cells of the synapse then give rise to a relayed system which enters the thalamus and tectum. Degeneration following lesions of the cervical cord would not pass beyond this synapse.

REFERENCES.

Adey, W. R., and Kerr, D. I. B. (1954): J. comp. Neurol., 100, p. 597.
Anderson, F. D., and Berry, C. N. (1959): *Ibid.*, 111, p. 195.
Bowsher, D. (1958): *Ibid.*, 110, p. 135.
Bowsher, D., Brodal, A., and Walberg, F. (1960): Brain, 83, p. 150.
Clark, W. E. Le Gros (1936): J. Anat., 71, p. 7.
Dennis, B. J., and Kerr, D. I. B. (1961): Austral. J. exp. Biol., 39, p. 43.
Ferraro, A., and Barrera, S. E. (1936): J. comp. Neurol., 64, p. 313.
Geberetzoff, M. A. (1939): La Cellule, 48, p. 90.
Getz, B. (1952): Acta Anat., 16, p. 271.
Goldby, F. (1943): J. Anat., 77, p. 195.
Häggquist, G. (1936): Ztschr. f. mikr.–anat. Forsch., 39, p. 1.
Kerr, D. I. B. (1961): In publication.
Matzke, H. A. (1951): J. comp. Neurol., 94, p. 439.
Mehler, W. R. (1957): Anat. Rec., 127, p. 332.
Nauta, W. J. H., and Kuypers, H. G. J. M. (1958): In "Reticular Formation of the Brain". Henry Ford Hospital International Symposium. (H. H. Jasper, Ed.) Little, Brown, Boston, p. 3.
Poirier, L. J., and Bertrand, C. (1955): J. comp. Neurol., 102, p. 745.
Ranson, S. W., and Ingram, W. R. (1932): *Ibid.*, 56, p. 257.
Swank, R. L., and Davenport, H. A. (1934): Stain Tech., 9, p. 11.
Wallenberg, A. (1900): Anat. Anz., 18, p. 81.

ABBREVIATIONS FOR ALL FIGURES

AF	arcuate fibres	MB	mammillary body
BC	brachium conjunctivum	MG	medial geniculate
BP	basis pedunculi	MLF	medial longitudinal fasciculus
CE	nucleus centralis	P	brachium pontis
CG	central grey	PF	nucleus parafascicularis
EML	external medullary lamina	Py	pyramids
F	fornix	RN	red nucleus
H	H fields of subthalamus	SC	superior colliculus
HP	habenulo-interpeduncular tract	SN	substantia nigra
IC	inferior colliculus	SO	superior olive
IO	inferior olive	T	trapezoid body
L	lesion	VP	nucleus ventralis posterior
LG	lateral geniculate	VPL	nucleus ventralis posterolateralis
LL	lateral lemniscus	III	third nerve
M	mammillo-thalamic tract	V	root of fifth nerve

SOMAESTHETIC PATHWAYS IN THE MARSUPIAL PHALANGER, *TRICHOSURUS VULPECULA*

by BARBARA J. DENNIS AND D. I. B. KERR

(From the Department of Human Physiology and Pharmacology, University of Adelaide).

(*Accepted for publication 22nd December, 1960.*)

SUMMARY.

Spinal afferent pathways corresponding to the classical somaesthetic projections, medial lemniscus and anterolateral column system, have been described for the marsupial phalanger, *Trichosurus vulpecula*, using potentials evoked by limb stimulation.

Potentials were also evoked within the central tegmental fasciculus and central grey which are considered to be phylogenetically older somaesthetic systems. These projections are relatively conspicuous in comparison with the classical paths, particularly the medial lemniscus. It is considered that the marsupial somaesthetic systems, with a dominance of medical indirect somaesthetic sensory paths, represent a step between the reptilian condition, where such paths predominate, and the full development of more direct spinothalamic projections in the higher mammals.

INTRODUCTION.

On the basis of evoked potentials four somaesthetic pathways have been described in the cat (Stotler and Kerr, 1955; Kerr *et al.*, 1955). These include the medial lemniscus which terminates in n. ventralis posterolateralis, and the anterolateral column system which projects to n. ventralis posterior. In addition, organised pathways were described within the central tegmental fasciculus and central grey matter.

As a comparative study, a search has been made for afferent systems in the Australian phalanger, *Trichosurus vulpecula*, no previous attempt having been made to elaborate the sensory pathways in this marsupial.

METHODS.

A stereotaxic atlas of the phalanger brain was prepared using alternate Nissl (Cresyl-violet) and fibre (Klüver, luxol-fast blue) sections cut at 50 μ. The horizontal plane of the cat stereotaxic instrument was modified by lowering the infra-orbital clamps 6 mm., thereby bringing the head into a position in which the plane of the vertical electrodes more nearly agreed with the true frontal plane.

The animals were anaesthetized with Dial (Ciba), the depth of anaesthesia being sufficient to eliminate flexor reflexes. Pulses, of 0·25 msecs. duration and supramaximal intensity, were applied to paired silver stimulating electrodes placed on isolated nerves of the fore and hind limbs, and the evoked potentials were led from the brainstem with conven-

tional preamplifiers and CRO recording. Recording electrodes were of insulated steel with an exposed tip of 30-50 μ (macroelectrodes) or 10-25 μ (semi-microelectrodes). An auditory monitor was included in the recording system.

At the conclusion of an experiment the animal was perfused with 10 p.c. formalin, ensuring that the brain was fixed at the instant of death. Histological verification of placement of electrodes was made, for all recording positions, in Nissl and Klüver stained sections.

RESULTS.

The medial lemniscus.

Using the technique of evoked potentials, the medial lemniscus could be traced from the medulla to a midhypothalamic level (Fig. 1). Fig. 2 shows a typical medial lemniscus response recorded at the midbrain. Electrical stimulation of isolated nerves in both fore and hind limbs evoked a response from the contralateral medial lemniscus. Light brushing of hairs on the limbs and paws was also found to be an adequate stimulus for evoked potentials in the lemniscus, particularly when using the auditory monitor.

At medullary levels just rostral to the main sensory decussation, a response could also be recorded from the ipsilateral side (Fig. 3); the latency of the ipsilateral potentials was similar to that of the contralateral response, while the amplitude was smaller. Acute section of the dorsal columns at C_2 abolished the ipsilateral as well as the contralateral potential. Marchi degeneration studies (Clezy *et al.*, 1961) lend support to the suggestion that some lemniscal fibres are still uncrossed at lower medullary levels. At more anterior levels there is no suggestion of bilateral representation in this pathway by any means of assessment.

Ascending the brainstem, in the frontal planes of the corpora quadrageṃina, the medial lemniscus runs just lateral to the interpeduncular nucleus. It persists in an essentially ventro-medial position in the midbrain and subthalamus until it sweeps fan-like from below into the n. ventralis posterolateralis, where it terminates as a thin ribbon lying above the external medullary lamina of the thalamus. Fig. 1 summarizes the regions from which lemniscal potentials could be recorded.

Within n. ventralis posterolateralis (level A13 in Fig. 1) the lemniscal termination showed a topographically-separated projection, the hindlimb representation lying laterally to the forelimb. Within the pathway itself the fibres of the lemniscus are spread as a thin band and it is difficult to assess any segmental representation.

The anterolateral system.

Bilateral potentials referable to the anterolateral system were traced from the level of the superior olives to the thalamus. Fig. 4 shows typical potentials recorded with the macroelectrode. In the medulla the fibres of the anterolateral column occupy the ventrolateral margin of the brainstem (level A1 in

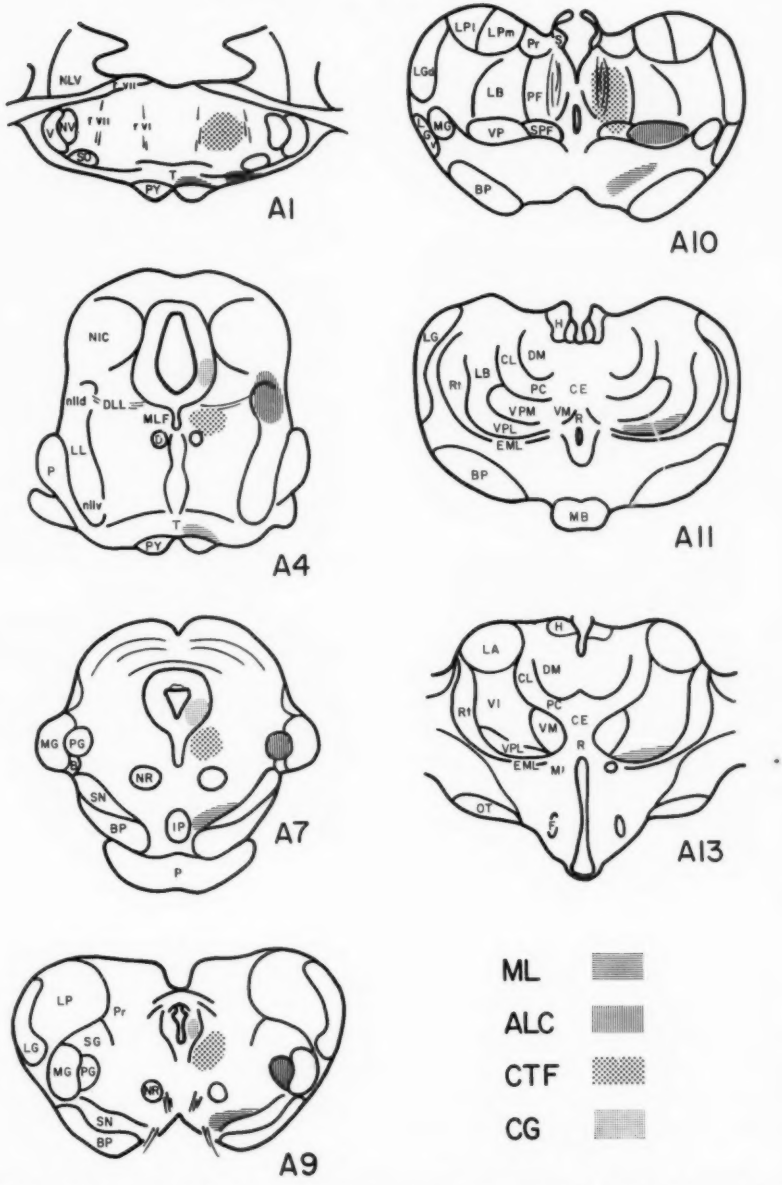


Fig. 1. *Regions from which somaesthetic responses were recorded in the phalanger brain.* The levels A1-A13 refer to Horsely-Clarke frontal planes. The key shows the shading used to indicate the extent of the various pathways.

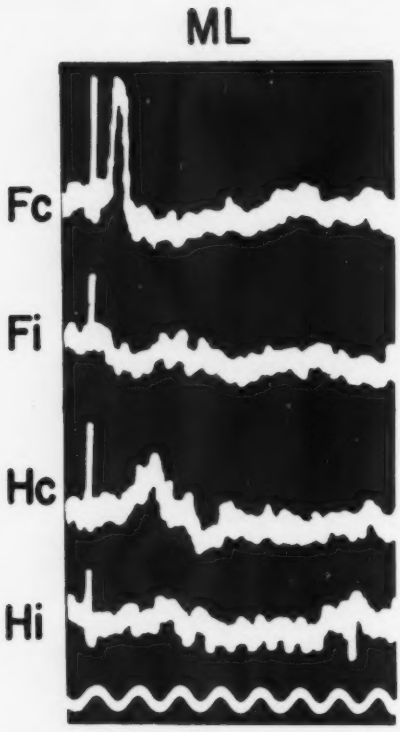


Fig. 2. *Potentials evoked in the medial lemniscus at the midbrain level.* Contralateral stimulation of forelimb or hindlimb yielded a response. Ipsilateral stimulation was ineffective. Time: 100 c.p.s. In this and subsequent figures Fc, Fi, Hc, Hi refer to contralateral and ipsilateral fore- and hindlimb stimulation respectively.

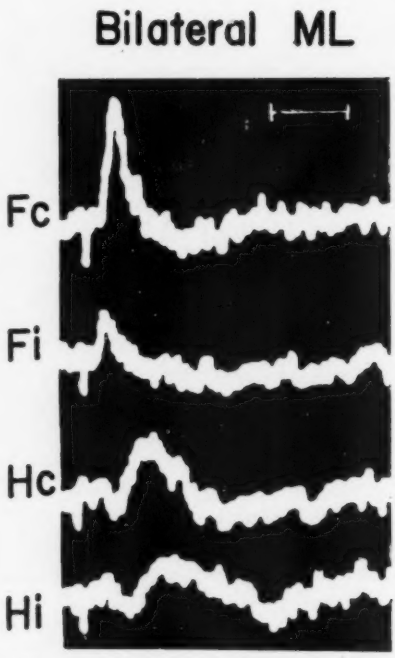


Fig. 3. *Bilateral potentials evoked in the medial lemniscus at a lower medullary level.* Time bar, 20 msecs.

Fig. 1) where Marchi degeneration (Clezy *et al.*, 1961) shows a bundle of degenerated fibres which are mainly those of the ventral spinocerebellar tract. The system could be traced through the inferior colliculus (level A4 in Fig. 1) into a region lateral to the central tegmental fasciculus and medial to the brachium of the inferior colliculus in the tectum (level A7). More rostrally, with the appearance of the medial geniculate bodies on the lateral surfaces of the brainstem, anterolateral responses are evoked from nucleus parageniculatus (Figs. 1 and 5), where they assume a spiking characteristic with semi-microelectrode recording (Fig. 6). This unitary activity is taken to be of synaptic origin and can be traced rostrally into n. ventralis posterior of the thalamus

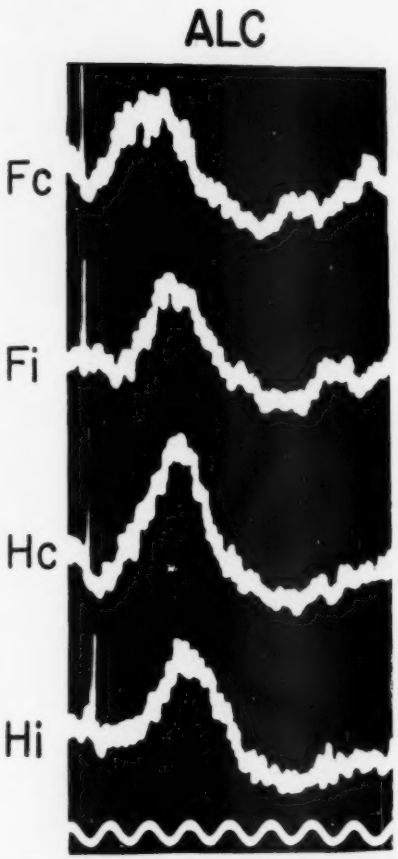


Fig. 4. *Responses evoked in the anterolateral system at a midbrain level.* Bilateral representation is characteristic of this system throughout its length. Time: 100 c.p.s.

(Fig. 1, level A10, and Fig. 9). It seems possible, therefore, on the basis of semi-microelectrode recording, that the termination of the anterolateral system takes place over some length of the brainstem in nuclei which are not obviously anatomically related. Furthermore, there is no obvious topographical separation within the termination, essentially the same unitary groups responding to stimulation of all four limbs (Fig. 6). This condition resembles that found in the cat by Poggio and Mountcastle (1960) in their "PO complex". Our n. parageniculatus is evidently homologous with the magnocellular division of the medial geniculate body (Whitlock and Perl, 1959). The greater part of the "PO complex" lies rather more rostrally, laterally and dorsally than n. ventralis posterior or n. parageniculatus.

While using auditory monitoring of peripherally-applied tactile stimuli it became obvious that the anterolateral system contained touch-responsive elements. Stroking the hairs of the paw was insufficient to elicit the response from the anterolateral system, but coarse mechanical stimulus (tapping on bony prominences) was adequate.

The central tegmental fasciculus.

Bilateral evoked potentials, with latencies longer than those of the anterolateral pathway, could be recorded from a medially placed afferent system (Fig. 7). These potentials were traced from the upper medulla to caudal thalamus (Fig. 1). At the level of the superior olive the potentials were found in a region 1-2 mm. dorsal to the anterolateral complex which lies on the ventrolateral surface of the medulla. The distinctness of the projections through the central tegmental fasciculus and anterolateral pathways is apparent since the

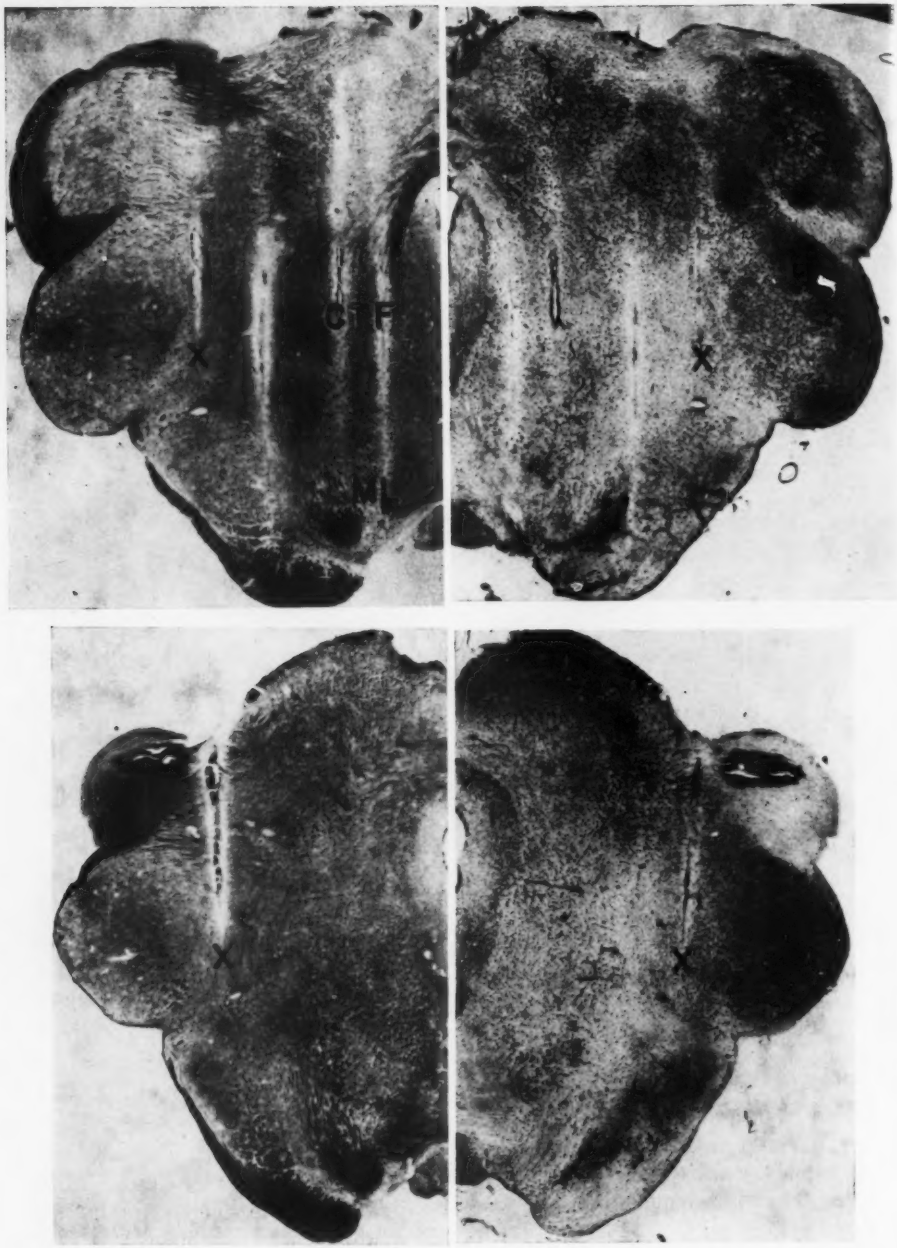


Fig. 5. *Photomicrographs of frontal sections through nucleus parageniculatus.* Nissl stain (on right) and Klüver fibre stain (on left) arranged in mirror image positions. The anterolateral system was traced to this nucleus. In the more rostral section (above) the most lateral electrode track penetrated an active area, marked X, yielding unitary responses; areas from which central tegmental fasciculus and medial lemniscus responses were recorded are indicated on the medial penetrations. At the superior collicular level (below) only one penetration was made and the electrode was not advanced beyond the strongly active area, marked X, yielding unitary responses.

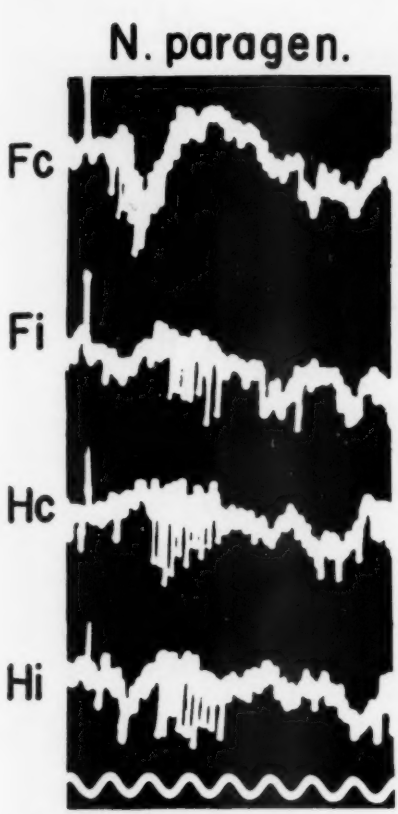


Fig. 6. *Unitary responses evoked in nucleus parageniculatus.* Semi-microelectrode recording yielded spiking responses of this type from n. parageniculatus throughout the regions indicated in Fig. 5. Time: 100 c.p.s.

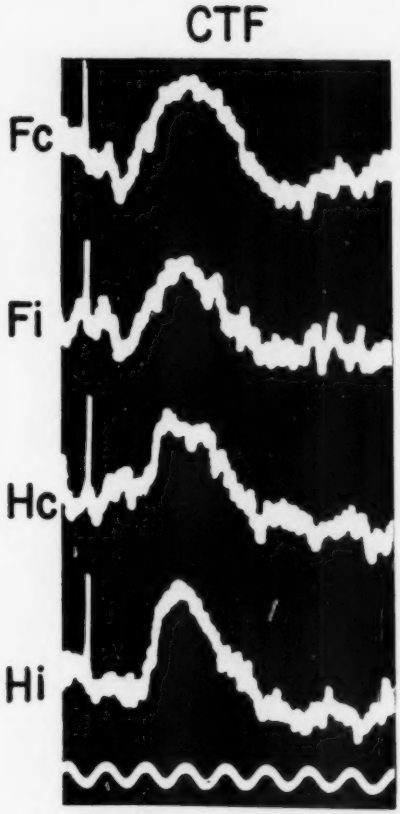


Fig. 7. *Bilateral potentials evoked in the central tegmental fasciculus recorded at a midbrain level.* The potentials have longer latencies than those of the anterolateral system. Time: 100 c.p.s.

two regions yielding evoked responses were separated by a region from which no afferent responses could be elicited, and the form of the two response patterns was quite distinct (Fig. 8).

The course of the central tegmental fasciculus through the midbrain is consistently ventrolateral to the central grey and medial to the anterolateral projection (Fig. 1). The system could be traced into n. parafascicularis of the midline thalamus nuclear complex. Fig. 9 shows potentials evoked from the terminating CTF, in nucleus parafascicularis (at lateral 1 mm.), and from the

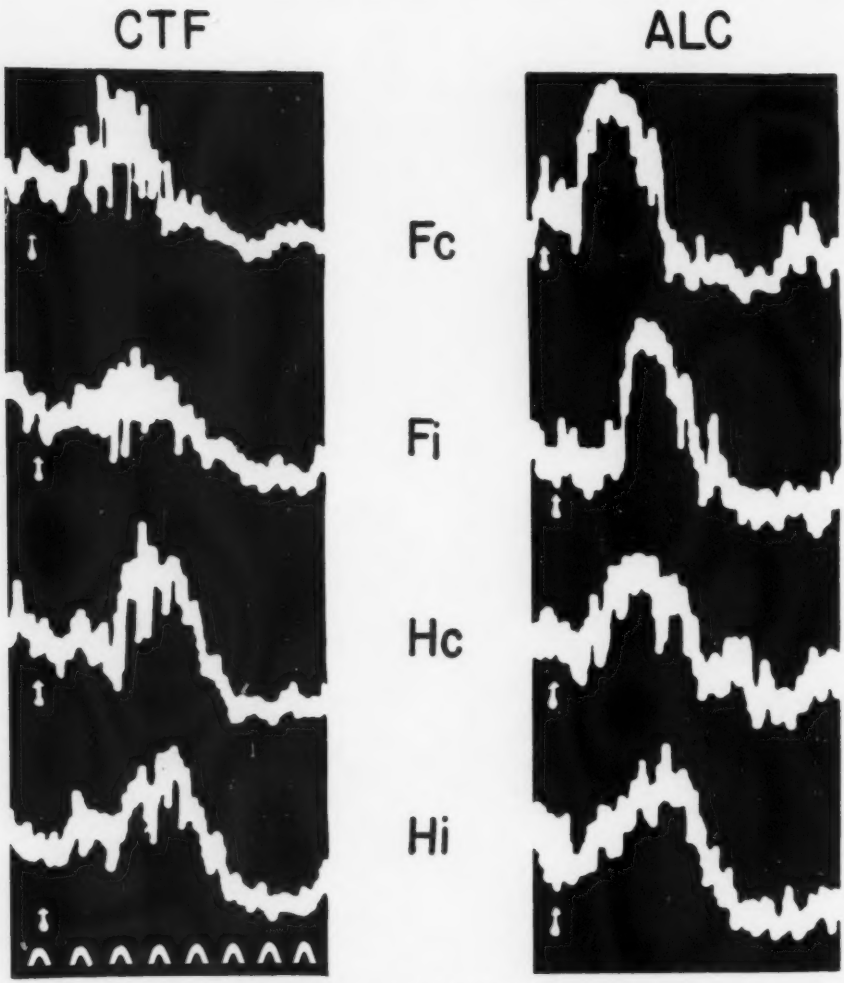


Fig. 8. *Comparison of evoked potentials in the central tegmental fasciculus and anterolateral system recorded at the superior olivary level.* The central tegmental fasciculus lies dorsal to the anterolateral system at this level. Evoked responses within it show more unitary spiking in comparison with the wave responses of the anterolateral system. Time: 100 c.p.s.

adjacent anterolateral system in nucleus ventralis posterior at lateral readings of 2, 3, 4 and 5 mm. (Horsely-Clarke level A10 in Fig. 1). The wave forms evoked from these two systems in the same experiment at the thalamic level, under the same recording conditions, were again quite different.

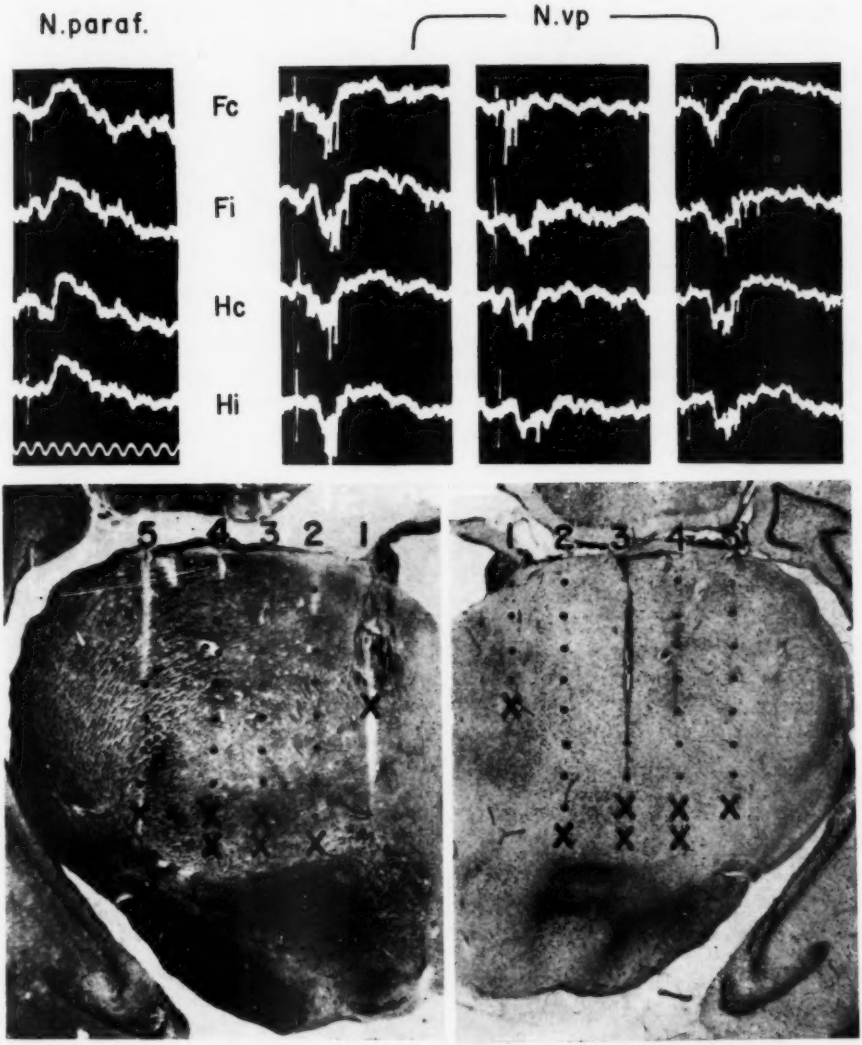


Fig. 9. *Comparison of evoked potentials recorded from the termination of the central tegmental fasciculus and the anterolateral system in the posterior thalamus.* The CTF gave positive-going wave responses in n. parafascicularis, the ALC gave unit discharges in n. ventralis posterior. The photomicrographs show the electrode penetrations from which the evoked responses were recorded. Adjacent cell and fibre stained sections have been arranged in mirror image position. The terminating CTF was recorded from rostral n. parafascicularis, at X in the most medial electrode penetration (lateral 1). In the four adjacent penetrations (lateral 2-5) responses from the anterolateral system were recorded from n. ventralis posterior, the active regions are indicated by crosses. (Evoked responses from lateral 2, 3 and 4 only are shown above.) Time: 100 c.p.s.

The central grey.

Recording at tectal levels disclosed potentials within the central grey. These potentials (Fig. 10) were traced to the periventricular thalamic region but their subsequent course has not been revealed. Similarities of latencies of the CG and CTF potentials may lead to confusion of these systems at rostral levels where the anatomic distinctness of the central grey has faded. More caudally the systems are quite separate, the central grey does not extend as far caudally as the central tegmental fasciculus. The afferent pathway to it is not known. Fig. 1 shows the positions from which central grey potentials could be recorded.

DISCUSSION.

The brainstem course and functional characteristics of these four afferent pathways in the phalanger are essentially similar to those found in the cat. Potentials recorded from the internal arcuate fibres illustrate the bilateral nature of the medial lemniscus in the lower medulla where crossing is achieved over several millimetres. In its passage from the rostral medulla to the thalamus the medial lemniscus is a completely contralateral projection. Termination of the lemniscus in nucleus ventralis posterolateralis is likewise in agreement with the state of affairs in other animals, Mountcastle and Henneman (1952), Poggio and Mountcastle (1960).

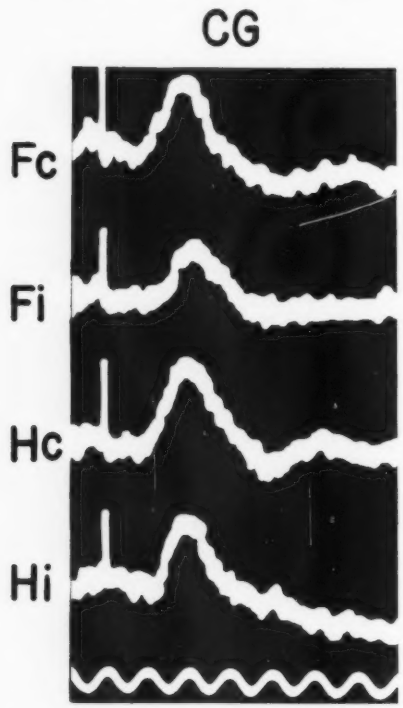


Fig. 10. *Bilateral evoked responses recorded from within the central grey of the tectum.* Time: 100 c.p.s.

There is a slight variation between the courses of the medial lemniscus in the cat and in the phalanger. In the cat the medial lemniscus overlies the substantia nigra at the level of the superior colliculus: it then bunches into a more compact bundle and migrates dorso-laterally at the level of the medial geniculate bodies so that the medial lemniscus and anterolateral systems merge in the posterior reaches of the thalamus. Consequently, responses evoked at this level in the anterolateral system

only become apparent after section of the dorsal columns. In the phalanger the lemniscus remains ventro-medially placed for a greater length of its ascent and at the level of the medial and lateral geniculate bodies persists as a ventro-medial band of fibres. Maintaining this relatively ventral position even at the level of n. ventralis posterior, termination above the externally medullary lamina in nucleus ventralis posterolateralis is then achieved by late migration of the lemniscus to a more dorsal position. This course is similar to that of the so-called aberrant lemniscal fibres occasionally encountered in the cat (Matzke, 1951).

Topographical separation of the evoked response is a feature of conduction in the medial lemniscus. The anterolateral, central tegmental fasciculus and central grey systems, however, do not display segmental separation when recording by these methods. Convergence of afferents from diverse sources upon a synaptic region giving rise to an "afferent final common path" would offer an explanation of this. Such convergence has been demonstrated in the anterolateral system (Dellow *et al.*, 1961) and in the central tegmental fasciculus (Dennis and Kerr, 1961).

The anterolateral system is evidently the homologue of the spinothalamic tract of primates but there is no anatomical evidence for a direct spinothalamic connection of large myelinated fibres in the phalanger. However, afferent volleys in the anterolateral system reach the thalamus with a velocity of conduction very little less than that of the myelinated medial lemniscus. This suggests conduction in fibres larger than those thought to constitute the marsupial anterolateral system, which is in any event poorly developed (Mehler, 1957). It may be supposed that a synapse within the pathway is responsible for apparent absence of myelinated fibres in the anterolateral projection above the medulla, as has been suggested for the same pathway in the cat (Stotler and Kerr, 1955; Kerr *et al.*, 1955; Dellow *et al.*, 1961).

In the phalanger the central tegmental fasciculus and central grey pathways are relatively larger, in comparison with the medial lemniscus and anterolateral system, than is the case in "higher" mammalian forms. Anatomically the pathways are well authenticated in mammals (Lewandowsky, 1904; Brodal and Rossi, 1955). It is therefore surprising that so little is known of their functional properties beyond there being some association of the central grey path with the response to noxious stimulation (Skultety, 1958; Melzack *et al.*, 1958), and of the central tegmental fasciculus with the medial ascending arousal system (Moruzzi and Magoun, 1949; Morin *et al.*, 1951; Russell, 1954; Brodal and Rossi, 1955).

Some insight into the possible significance of these medial paths can be gained from a consideration of their place in the phylogeny of the somaesthetic systems and the thalamus. Primitively, in the lower vertebrates, sensory impulses arrive at the diencephalon essentially after relaying in the tectum, and connections with the epithalamus predominate. The epithalamus remains re-

latively static and it is the thalamus proper that undergoes progressive elaboration in phylogeny. As this occurs, the beginnings of more direct sensory pathways from the cord to the diencephalon become apparent, always, however, with their passage interrupted at an integrative relay in the bulb (Herrick, 1948).

At the evolutionary stage of the reptilian brain, somatic parts of the thalamus appear more definitely differentiated. In particular, two nuclear groups with somatic connections may be discerned; these are n. rotundus (and n. dorsolateralis anterior, not entirely differentiated from the latter) and n. reuniens.[1] There is some confusion concerning the homology of these two groups with the ventral posterior somaesthetic complex of mammals. There are grounds for considering that the reptilian n. rotundus is the *anlage* of the mammalian ventral posterior groups, whilst the reptilian central group (n. reuniens) persists as the midline nuclei (Ingvar, 1923; Huber and Crosby, 1926; Clark, 1932; Papez, 1936; Kuhlenbeck, 1937; Walker, 1938). In any event, both these nuclear groups have largely striatal connections since the reptile has very little development of cortical fields to which the thalamus might project.

The midline nuclei of mammals retain connections with the lenticular nuclei, and also project to the basal telencephalon (Nauta and Whitlock, 1954; Powell and Cowan, 1954; Nauta and Kuypers, 1958; McKegney, 1958). In the marsupial, n. parafascicularis is the most conspicuous caudal nucleus of the medial thalamus. Rostrally it comes into contact with n. centralis, n. reuniens and n. ventralis medialis. These medial nuclei are in turn interconnected by intrathalamic fibres, and project, through n. ventralis medialis, to the lenticular nuclei. For this reason we are inclined to consider that n. parafascicularis represents a functional remnant of the reptilian nucleus reuniens in that it receives a somatic input over a medial pathway derived from lower brainstem sensory relays. The central tegmental fasciculus in turn represents the somatic portion of the old sensory fasciculi derived from bulbar sensory relays, which appear in phylogeny before the more direct lemniscal systems (Herrick, 1948).

The central grey path presumably represents the old afferent projection to the pars ventralis of the diencephalon, which is quite apparent in the amphibian brain (Herrick, 1948).

The growth of the ventral thalamus in phylogeny is reflected in the relatively small dimensions of n. ventralis posterior and n. ventralis posterolateralis as they appear in the marsupial by comparison with the thalamus of a carnivore. In particular, the medial lemniscus is only poorly developed in the marsupial. This is also emphasized by the relatively small cortical afferent projection of the limbs on to somatic area 1 of the phalanger cortex (Adey and Kerr, 1954).

---

[1]It is unfortunate that the term "nucleus reuniens" occurs in descriptions of both mammalian and reptilian brains. As indicated above, the reptilian n. reuniens probably elaborates into the centralis complex and intralaminar nuclei of mammals.

The region which has been designated nucleus parageniculatus in the phalanger (Fig. 1) is homologous with the magnocellular division of the medial geniculate of higher forms in that it receives the anterolateral column pathway, with the difference that it lies external to the brachium of the inferior colliculus. This nucleus may in part receive a tecto-thalamic path (tractus tecto-reuniens); however, we consider that this region is not part of n. subparafascicularis as was suggested by Bodian (1939), Goldby (1941) and Adey *et al.* (1958). The connections of n. parageniculatus with the anterolateral system seem to be direct rather than through the tectum at least so far as can be assessed from latencies of evoked potentials. The thalamic parafascicular complex lies more medially and receives the central tegmental fasciculus projection which is a separate pathway from the anterolateral system. The anterolateral system may be traced caudally from the thalamus into the inferior colliculus below which it is seen to join the ventral spino-cerebellar division of the anterolateral projection (Fig. 1, and Figs. 11-20 in Clezy *et al.*, 1961).

REFERENCES.

Adey, W. R., Dunlop, C. W., and Sunderland, S. (1958): J. comp. Neurol., 110, p. 173.

Adey, W. R., and Kerr, D. I. B. (1954): *Ibid.*, 100, p. 597.

Bodian, D. (1939): *Ibid.*, 71, p. 259.

Brodal, A., and Rossi, G. F. (1955): A.M.A. Arch. Neurol. Psychiat., 74, p. 68.

Clark, W. E. Le Gros. (1932): Brain, 55, p. 406.

Clezy, J. K. A., Dennis, Barbara J., and Kerr, D. I. B. (1961): Austral. J. exp. Biol., 39, p. 19.

Dellow, P. G., Dennis, Barbara J., and Kerr, D. I. B. (1961): To be submitted for publication.

Dennis, Barbara J., and Kerr, D. I. B. (1961): Austral. J. exp. Biol., 39, p. 43.

Goldby, F. (1941): J. Anat., 75, p. 197.

Herrick, C. J. (1948): "The Brain of the Tiger Salamander, *Ambylstoma tigrinum*". University of Chicago Press, Chicago.

Huber, G. C., and Crosby, E. C. (1926): J. comp. Neurol., 40, p. 97.

Ingvar, S. (1923): Acta Medica Scand., 59, p. 696.

Kerr, D. I. B. (1961): To be submitted for publication.

Kerr, D. I. B., Haugen, F. P., and Melzack, R. (1955): Amer. J. Physiol., 183, p. 253.

Kuhlenbeck, H. (1937): J. comp. Neurol., 66, p. 23.

Lewandowsky, M. (1904): In: O. Vogt. Neurobiol. Arbeiten II. Serie, Bd. 1.2, p. 63.

McKegney, F. P. (1958): Yale J. Biol. Med., 30, p. 415.

Matzke, H. A. (1951): J. comp. Neurol., 94, p. 439.

Mehler, W. R. (1957): Anat. Rec., 127, p. 332.

Melzack, R., Stotler, W. A., and Livingston, W. K. (1958): J. Neurophysiol., 21, p. 353.

Morin, F., Schwartz, H. G., and O'Leary, J. L. (1951): Acta Psychiat. Kbh., 26, p. 371.

Moruzzi, G., and Magoun, H. W. (1949): EEG and clin. Neurophysiol., 1, p. 455.

Mountcastle, V. B., and Henneman, E. (1952): J. comp. Neurol., 97, p. 409.

Nauta, W. J. H., and Kuypers, H. G. J. M. (1958): In: "Reticular Formation of the Brain". Henry Ford Hospital Symposium. (H. H. Jasper, Ed.) Little, Brown. Boston, p. 3.

Nauta, W. J. H., and Whitlock, D. G. (1954): In: "Brain Mechanisms and Consciousness". A symposium. (J. F. Delfresnaye, Ed.) Blackwell, Oxford, p. 81.

Papez, J. W. (1936): J. comp. Neurol., 64, p. 41.

Poggio, G. F., and Mountcastle, V. B. (1960): Johns Hopk. Hosp. Bull., 106, p. 266.

Powell, T. P. S., and Cowan, W. M. (1954): J. Anat., 88, p. 237.

Russell, G. V. (1954): J. comp. Neurol., 101, p. 237.
Skultety, F. M. (1958): *Ibid.*, 110, p. 337.
Stotler, W. A., and Kerr, D. I. B. (1955): Anat. Rec., 121, p. 418.
Walker, A. E. (1938): "The Primate Thalamus". University of Chicago Press, Chicago.
Whitlock, D. G., and Perl, E. R. (1959): J. Neurophysiol., 22, p. 133.

ABBREVIATIONS FOR ALL FIGURES.

ALC	anterolateral column pathway (anterolateral system).
B	brachium of the inferior colliculus.
BP	basis pedunculi.
CE	nucleus centralis.
CG	central grey afferent pathway.
CL	nucleus centralis lateralis.
CTF	central tegmental fasciculus.
D	dorsal tegmental nucleus of Gudden.
DLL	decussation of the lateral lemniscus.
DM	nucleus dorsomedialis.
EML	external medullary lamina.
F	fornix.
H	habenula.
IP	interpeduncular nucleus.
LA	nucleus lateralis A (Goldby).
LB	nucleus lateralis B (Goldby).
LG	lateral geniculate body.
LGd	dorsal nucleus lateral geniculate.
LGv	ventral nucleus lateral geniculate.
LL	lateral lemniscus.
LP	nucleus lateralis posterior.
LPl	nucleus lateralis posterior, pars lateralis.
LPm	nucleus lateralis posterior, pars medialis.
M	mammillo-thalamic tract.
MB	mammillary body.
MG	medial geniculate body.
ML	medial lemniscus.
MLF	medial longitudinal fasciculus.
NIC	nucleus of inferior colliculus.
nlld	dorsal nucleus of the lateral lemniscus.
nllv	ventral nucleus of the lateral lemniscus.
NLV	lateral vestibular nucleus.
NR	red nucleus.
NV	nucleus of spinal tract of trigeminal nerve.
OT	optic tract.
P	pons, brachium pontis.
PC	nucleus paracentralis.
PF	nucleus parafascicularis.
PG	nucleus parageniculatus.
Pr	nucleus pretectalis.
Py	pyramids.
R	nucleus reuniens.
Rt	reticular nucleus of thalamus.
rvi	radix sixth nerve.
rvii	radix seventh nerve.
S	stria medullaris.
SG	nucleus suprageniculatus.
SN	substantia nigra.
SO	superior olive.
SPF	nucleus subparafascicularis.
T	trapezoid body.
V	root of fifth nerve.
Vl	nucleus ventralis lateralis.
VM	nucleus ventralis medialis.
VP	nucleus ventralis posterior.
VPL	nucleus ventralis posterolateralis.
VPM	nucleus ventralis posteromedialis.

AN AFFERENT SYSTEM IN THE CENTRAL TEGMENTAL FASCICULUS

by BARBARA J. DENNIS AND D. I. B. KERR

(From the Department of Human Physiology and Pharmacology, University of Adelaide).

(*Accepted for publication 22nd December, 1960.*)

SUMMARY.

The brainstem course of the limb afferent component of the central tegmental fasciculus has been determined in both the cat and the phalanger, *Trichosurus vulpecula*, using evoked potentials. Unit records obtained from the reticular formation indicate that the ascending central tegmental fasciculus arises in large part from the level of the superior olives. The termination of the system is found mainly in n. parafascicularis of the posterior thalamus.

Attention is drawn to the correspondence between the ascending central tegmental fasciculus and the reticular activating system (arousal system) of the medial brainstem.

INTRODUCTION.

"In 1877 Forel described a group of bundles which differentiate from the reticular formation in the most caudal cross sections of the anterior quadragemina bodies and which he denoted as the tegmental fasciculi ('Haubenfascikel'). He could follow it into the thalamus, but its exact terminations, in particular a change in its course, he was unable to demonstrate. If I correctly understand Wallenberg, the author then lets his crossed central trigeminus tract in the rabbit pass through these bundles, without, however, mentioning Forel. Ultimately Hatcheck depicted the topography of these bundles in a number of species, but I myself am of the opinion that the illustrations of Hatcheck are nothing else than the fasciculus of Forel, which he was able to follow downwards.

"Hatcheck himself seems to have considered Forel's fasciculus to be not identical with his bundles which he, without proof, described as a central Vth pathway.

"The central Vth pathway has certainly nothing to do with Forel's fasciculus. I have at my disposal very extensive destructions of the spinal trigeminal root without any trace of degeneration of the crossed fasciculus. Furthermore, the fasciculus of Forel in the cat and dog is an exclusively uncrossed pathway. That some few fibres cross first in the raphé before they join the longitudinal fibres of the fasciculus in the rabbit, I do not with certainty wish to deny.

"The area of origin of Forel's fasciculus is very extensive. It extends caudally as far as the area of the inferior olive. The fibres originating caudally take a very random course in the reticular formation laterally from the tractus longitudinalis posterior, to arrive at the ventral surface of the nucleus dorsalis acoustica. Here they still lie between the Abducens nerve and descending Facial nerve, ventral to the genu of the Facial (Fig. 6). On the way to this point this pathway receives new increment. I am in fact under the impression, however, that this fasciculus takes up the major part of its fibres just here, or still further rostrally in the pons. For in the region of the posterior quadragemina the fibres of Forel's fasciculus form a very prominent area in the dorsal section of the reticular formation, dorsal and close to the brachium conjunctivum before its crossing, and close to the central grey (Fig. 13). In the plane of the anterior quadragemina we then have the picture as described by Forel (Fig. 14). Somewhat further rostrally, corresponding to Meynert's plane of the mammillary bodies, the dorsal ascending fibres of the lemniscus principalis break through the ranks of the Lateral Fasciculi of Forel, as Forel himself described. Shortly afterwards the fibres then turn characteristically in a ventrolateral direction and terminate by spreading out in a field which is prominent always by its brightness in Weigert as well as Marchi preparations, and is situated medially from nucleus ventralis c, ventral from medialis b, and dorsally from field H. This field corresponds to a part of campus supra-reticularis of the Vogts. Its grey, in which we therefore demonstrated the termination of Forel's fasciculus, we will designate the Grey of Forel's Fasciculus (Fig. 15).

"The origin of the pathway is unfortunately less easy to detect. We do not know from which cells of the reticular formation of the oblongata or pons this pathway arises. The statement by Probst that it is partly from the cerebellum will be contested in an appropriate place."

In this way Lewandowsky (1904) described his "Tractus Fasciculorum Foreli", which is now conveniently referred to as the central tegmental fasciculus[1] (CTF). This fasciculus can be recognised in a wide variety of species and is a mixed fibre bundle containing ascending and descending components from various sources. In the lower medulla it is found as an inconspicuous system adjacent to the lateral aspect of the inferior olives. At the rostral medullary and pontine levels it organises into a major fibre grouping within which various subdivisions may be recognised. In the midbrain the CTF lies ventrolateral to the periaqueductual grey matter, occupying considerable proportion of the brainstem reticular formation.

The ascending and descending components to some extent can be distinguished in brains taken from cases of unilateral cerebellar agenesis. In a "system agenesis" of this type most of the pathways and structures functionally associated with the cerebellum are developed in relation to the intact side only. The

[1] The system is also called: Fasciculus centralis tegmenti (Bechterew), Zentrale Haubenbahn, and tractus tegmentalis centralis.

tegmental tracts joining the midbrain structures and inferior olives are thus missing on one side of such a brain, and the central tegmental fasciculus is consequently reduced on the affected side (Fig. 1). The remaining fibres of the fasciculus adjacent to the central grey of the midbrain are mostly ascending.

The ascending systems are considered to be reticulothalamic (Russell, 1954; Brodal and Rossi, 1955), medial spino-thalamic (Anderson and Berry, 1959; Mehler, 1957; Nauta and Kuypers, 1958), and trigemino-thalamic (Torvick, 1957; Carpenter, 1957). A component of the superior cerebellar peduncle (McMasters and Russell, 1958) may also be present in normal material.

The precise thalamic termination of these various ascending paths of the medial brainstem is still uncertain, but n. ventralis posteromedialis, n. centre median, the parafascicularis complex and midline nuclei, and the intra-laminar thalamic nuclei have been indicated as probable termination sites. Their various origins are also somewhat undecided, but a significant proportion arise from the pontine and upper-medullary reticular formation (Brodal and Rossi, 1955).

The present report outlines the origin, course and termination of an afferent component in the central tegmental fasciculus which can be activated by stimulation of limb nerves, and indicates some functional characteristics of the pathway.

Methods.

Cats and phalangers (*Trichosurus vulpecula*) were prepared under pentobarbital (Sagatal, May and Baker) or Dial (Ciba) anaesthesia. The brainstem was exposed for stereotaxic orientation of monopolar pick-up needles insulated to the tip, with a variety of tip sizes. The smallest needles were prepared from sewing needles by electrolytic etching of the tip and were suitable for recording unit activity from the brainstem.

Conventional R-C coupled amplifiers and oscilloscopes were used for recording and display of the evoked potentials. Tetronix and Grass stimulators were used to deliver supramaximal stimuli to exposed infraorbital, superficial radial and sural nerves. The brainstem was systematically explored for evoked responses from the inferior olivary level up to the rostral limit of n. ventralis posterolateralis of the thalamus.

RESULTS.

Brainstem course of the CTF.

The course of the ascending (afferent) component of the central tegmental fasciculus as defined electrophysiologically has been described previously (Dennis and Kerr, 1961, phalanger; Kerr, 1961, cat), and only requires brief repetition here with more detailed reference to the origin of the system. From a region in the medulla dorsal to the anterolateral column pathway (Gower's tract), adjacent to the dorsal aspect of the inferior olives and lying between the VIth and VIIth nerves, sensory evoked responses were recorded which showed a characteristic negative spiking with semi-microelectrode recording. Negative spikes appeared either alone or superimposed on a slow positive wave (the bandpass of the amplifier admits both waveforms) (Figs. 2, 3). This wave-

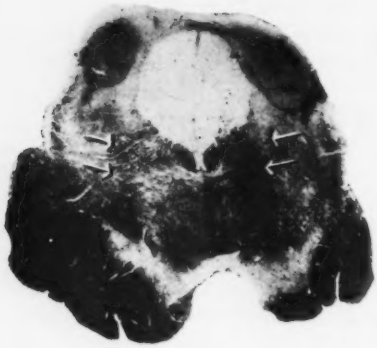


Fig. 1. The afferent central tegmental fasciculus (CTF) in a case of unilateral cerebellar agenesis. The CTF, indicated by the arrows, is markedly reduced on the right side of the brain with its absent cerebellar and associated connections. Weil Stain, Human. (Courtesy of Dr. W. A. Stotler, University of Oregon.)

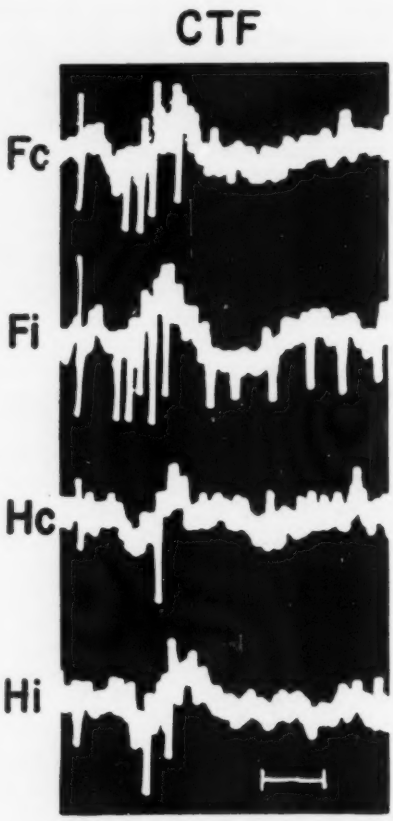


Fig. 2. Unit discharge from the origin of the CTF in the reticular formation at the level of the superior olives. Stimulation of Fc, Fi, Hc, Hi: contralateral and ipsilateral forelimb and hindlimb respectively. Phalanger. Time bar: 20 msecs.

form, with its spiking, was quite different from that encountered at the margin of the brainstem within the anterolateral system which yielded only rounded positive-going waves following limb afferent stimulation (see Fig. 8 of Dennis and Kerr, 1961). The two systems could also be distinguished in the brainstem on the basis of the latencies of the sensory evoked responses (6-8 msecs. ALC; 8-10 msecs. CTF with contralateral forelimb stimulation).

Below the superior olivary level no corresponding organised medial source of spiking potentials was found, but a considerable anterolateral column projection was encountered more laterally. In Marchi studies a medial group of fibres of spinal origin is found adjacent to the marginal anterolateral system at the inferior olivary level. This medial group persists as a dorsal extension of the anterolateral system at the level of the facial nucleus and superior olives where the degeneration appears as fine Marchi granules, indicating that these

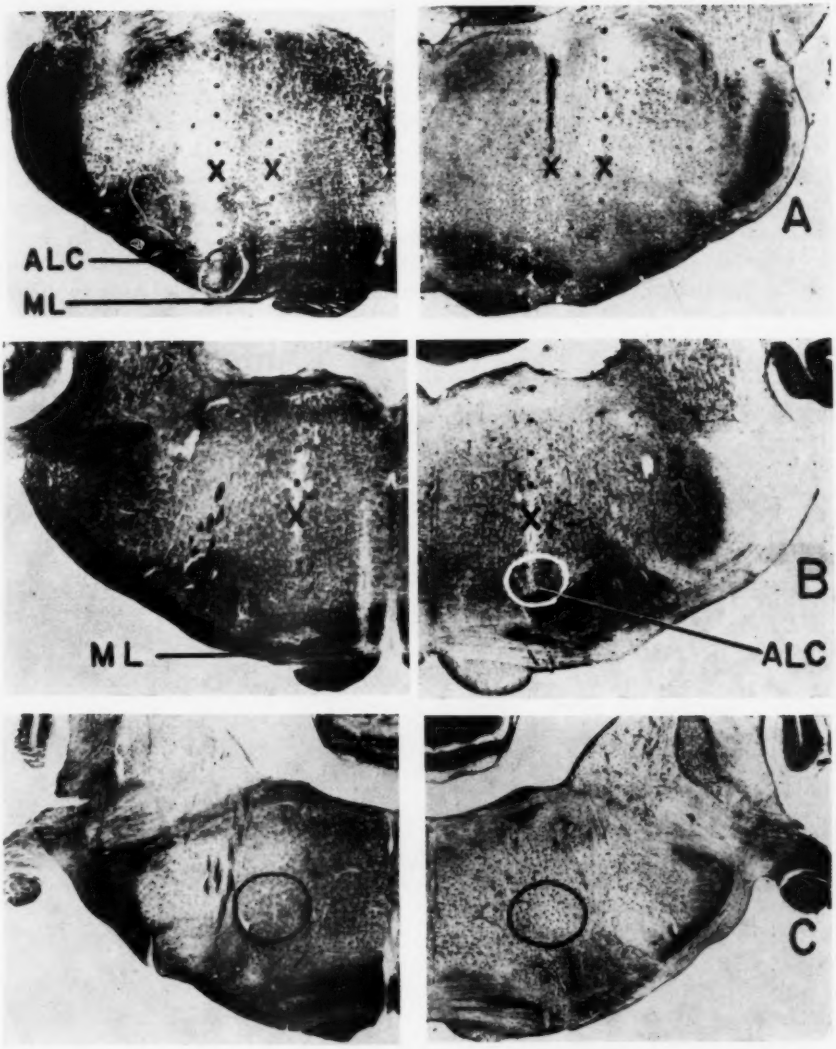


Fig. 3. Origin of the CTF in the phalanger. Photomicrographs of the brainstem at the level of the superior olives. Nissl (right) and fibre (left) stained sections arranged as mirror images. A. The maximum unitary focus was at X in both electrode penetrations. The medial penetration reached the medial lemniscus (ML) on the lateral margin of the pyramid; the lateral penetration reached the anterolateral system (ALC) encircled. B. More caudally the focus was again at X. The lateral penetration was halted in the medial division of the anterolateral system (Clezy *et al.*, 1961) adjacent to the inferior olive. The medial electrode reached the medial lemniscus but passed too medially for recording reticular activity in the CTF. C. The encircled area indicates the focus of maximal reticular unitary activity evoked by limb afferent stimulation, based on the superimposed results from a number of animals.

fibres are losing their myelin sheaths near a synapse (Clezy *et al.*, 1961). The synaptic area suggested by the Marchi method is close to that indicated by unitary activity in the evoked sensory responses, although the Marchi method is not suitable for demonstrating terminations.

Fig. 3A shows two foci of maximal evoked unitary spiking recorded at a rostral medullary level. The foci are indicated by X along the needle penetrations. At the base of the medial penetration the medial lemniscus was encountered. The anterolateral system was similarly found in the encircled region where the lateral needle penetration reached the ventral surface of the brain. In a needle penetration at a more caudal level (Fig. 3B) spiking was maximal at the region X. Ventral to this there was an area, indicated by a circle, which

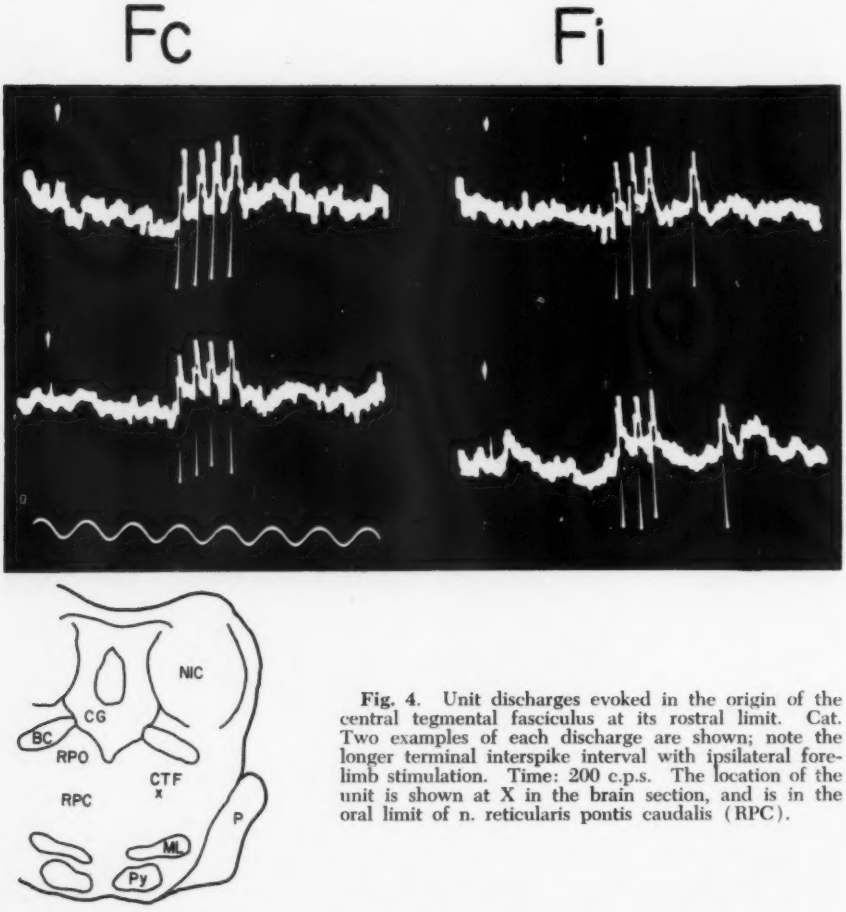


Fig. 4. Unit discharges evoked in the origin of the central tegmental fasciculus at its rostral limit. Cat. Two examples of each discharge are shown; note the longer terminal interspike interval with ipsilateral forelimb stimulation. Time: 200 c.p.s. The location of the unit is shown at X in the brain section, and is in the oral limit of n. reticularis pontis caudalis (RPC).

yielded wave responses. These probably came from fibres of the spino-reticular component of the anterolateral system where these penetrate into the reticular formation adjacent to the superior olive. On this occasion the recording needle was not introduced into the main anterolateral projection which lies ventral to the olives. The encircled area in Fig. 3C shows the extent of the reticular formation from which spiking was recorded with limb afferent stimulation at the level of the VIIth and VIth nerve roots in the medulla, and is based on the superimposed data of a number of experiments.

The zone of spiking afferent potentials extended into the pontine tegmentum as far as the oral limit of n. reticularis pontis caudalis (Fig. 4). The responses evoked along the course of the central tegmental fasciculus lost their spiking character and became positive-going waves at the inferior collicular level (n. reticularis pontis oralis) where the fasciculus is organised into a discernible tract (Fig. 5). The waveform then remained quite similar, up to the level of termination in the thalamus (Figs. 6, 8, 9). The region from which the potentials were evoked lay consistently within the boundaries of the central tegmental fasciculus, ventrolateral to the central grey, lateral to the medial longitudinal fasciculus, medial to the anterolateral system and dorsal to the tegmental decussation (at inferior collicular levels), dorsal to the decussation of the brachium conjunctivum (at superior collicular levels), and dorsal to the red nucleus (at geniculate levels). It could be traced into nucleus parafascicularis of the posterior thalamus. A curious feature of this termination was the absence of unitary spiking so characteristic of the anterolateral system in n. ventralis posterior and n. paragenicularis (see Fig. 9 in Dennis and Kerr, 1961).

Fig. 5. Evoked potentials were recorded from the central tegmental fasciculus at the position marked X on the fibre stained section taken at the inferior collicular level where the fasciculus is organised into a fibre tract beneath the central grey. The anterolateral system is more laterally placed within the confines of the lateral lemniscus. Phalanger.

In the posterior thalamus of the phalanger n. parafascicularis is a conspicuous nucleus which embraces the dorsal half of the habenulo-interpeduncular tract; it is relatively less developed in the cat. The central tegmental fasciculus is mainly distributed to the lateral segments of the nucleus but responses were also recorded from the medial segment immediately adjacent to the tract. In the experiment illustrated in Fig. 6 the plane of the needle penetrations corresponded with that of the fibres of the habenulo-interpeduncular tract. The needle penetration at L2 entered n. parafascicularis and was not car-

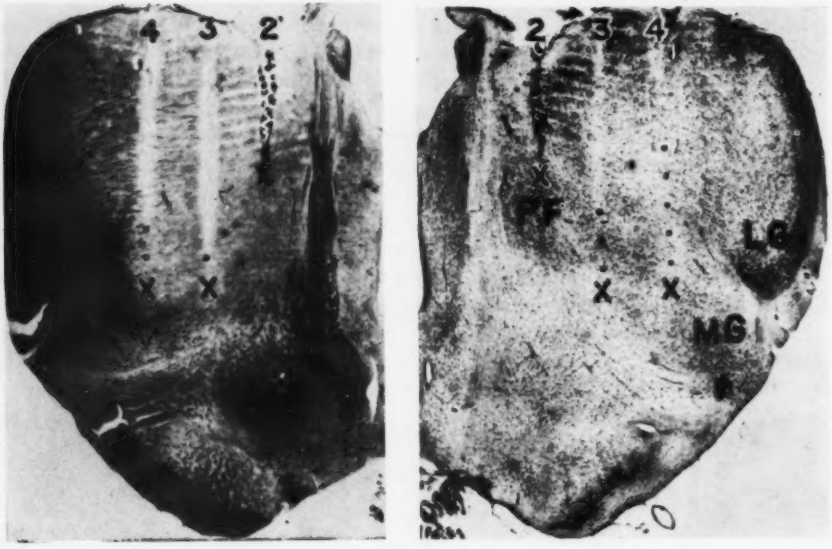


Fig. 6. The central tegmental fasciculus termination in n. parafascicularis adjacent to the habenulo-interpeduncular tract. Phalanger. The needle penetration was halted at a maximal response recorded at X on L2 penetration. At the base of penetrations L3, 4 the anterolateral system was recorded. In L3 the active region X lies in the caudal reaches of n. ventralis posterior. In L4 the needle recorded from X in n. parageniculatus at its rostral limit where the medial geniculate (MG) begins to give way to the lateral geniculate nucleus (LG).

ried deeper. The responses of Fig. 7 were recorded from the region X at the base of the penetration. This region is clearly separated from the anterolateral projection which was encountered more ventrally, at L3 and L4 in the most caudal extent of n. ventralis posterior (X in penetration L3) and the rostral extent of n. parageniculatus (X in penetration L4), overlying the subthalamus.

Convergence and interaction in the CTF.

Referring again to Figs. 2 and 4, it will be seen that the neuronal units yielding spike potentials were repeatedly activated regardless of the limb stimulated. This is taken as indicating convergence of afferent fibres of several origins upon a common synaptic relay in the pathway, since we were here recording from a single cell or a very synchronised group of cells. It is of interest that the firing pattern for unitary activation from the two sides of the body differed in a consistent fashion (Fig. 4) with a longer terminal interspike interval following ipsilateral stimulation. The responses shown here were taken some sweeps apart. Failure of the unit, due to damage with the recording needle, was indicated by a progressive lengthening of the last interspike interval of the discharge evoked by ipsilateral stimulation. This last spike then failed,

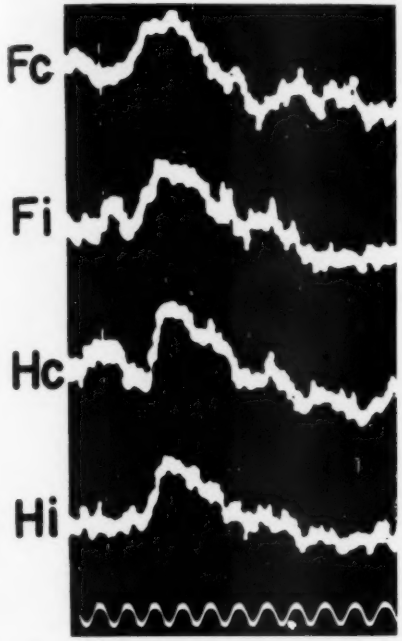


Fig. 7. Potentials evoked in n. parafascicularis by stimulation of Fc, Fi, Hc, Hi (contralateral and ipsilateral fore and hindlimbs respectively). Time: 100 c.p.s. Phalanger.

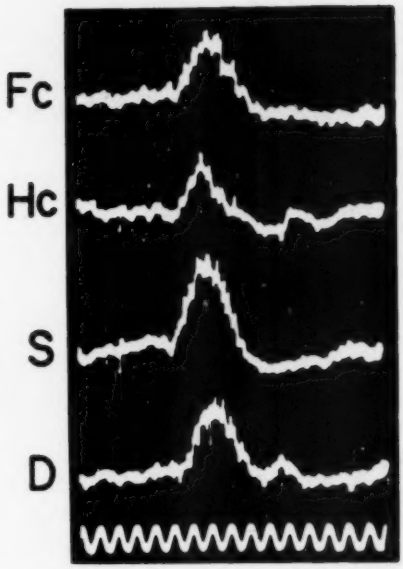


Fig. 8. Interaction and summative convergence in the central tegmental fasciculus. Cat, recorded at a midbrain level. Fc, contralateral forelimb, and Hc, contralateral hindlimb, evoked responses summated (S) when coincident at the recording needle. The Hc response was depressed (D) when it followed Fc by 20 msecs. Time: 200 c.p.s.

and the unit responded only three times to the incoming volley. Finally, after a brief period of spontaneous discharge, it abruptly ceased to respond to afferent stimulation. All of this we take to be evidence that the unit responses were recorded from a large cell of n. reticularis pontis caudalis (Fig. 4).

Further evidence for convergence in the bulbar synaptic relay of the CTF was obtained by interacting responses, recorded from the system at the midbrain level, when evoked by stimulation of different body regions. Fig. 8 shows the results of interacting CTF responses evoked by contralateral forelimb and contralateral hindlimb stimulation. If the two responses coincided in time at the pick up needle, i.e. the hindlimb was stimulated prior to the forelimb to allow for the longer latency of the former, the responses summated to give a larger wave (Fig. 8S). When stimulation of the hindlimb was moved to occur

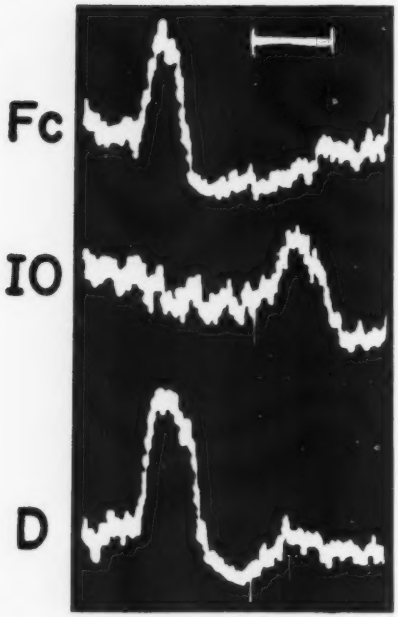


Fig. 9. Interaction between limb and trigeminal components of the central tegmental fasciculus. Cat, recorded at a midbrain level. The response to contralateral infraorbital stimulation (IO) was depressed (D), when it followed the contralateral forelimb response (Fc) by 50 msecs. Time bar: 25 msecs.

after forelimb stimulation, there was virtual abolition of the hindlimb response which now occurred after the peak of the forelimb response (Fig. 8D).

The trigeminal component of the central tegmental fasciculus (Kerr *et al.*, 1955) exhibited very similar interactions when interplayed with the limb component. In Fig. 9 when a contralateral forelimb response conditioned an infra-orbital response the latter was almost abolished. This indicates that the trigeminal and limb components also converge at the relay, but so far no unit records of this convergence are available.

This kind of interaction with summation and depressive interaction, depending on the relative arrival times of responses in the brainstem, can best be explained in terms of convergence and occlusion of afferents on a common synaptic relay. The unit records described above indicate that for the central tegmental fasciculus this relay is in the reticular formation of the upper medulla and pons.

DISCUSSION.

Although the functional significance of the central tegmental fasciculus as an afferent pathway is not known, it is of considerable phylogenetic antiquity with its ultimate connections directed towards the striatum and basal telencephalon (Kerr, 1961; Dennis and Kerr, 1961). In its functional properties the CTF bears a close resemblance to the Reticular Activating (Arousal) System (RAS) of Magoun (1952). Actually it more closely resembles the collateral afferent reticular projection (CAR) which is partly coincident with the arousal system (Starzl *et al.*, 1951b). Evoked potentials in both the CTF and CAR have longer latencies than the lemniscal pathways. There is also a prolonged period (almost 100 msecs.) of depressed conduction following a conditioning shock to an afferent nerve in both the CTF (Kerr, 1961) and CAR (French

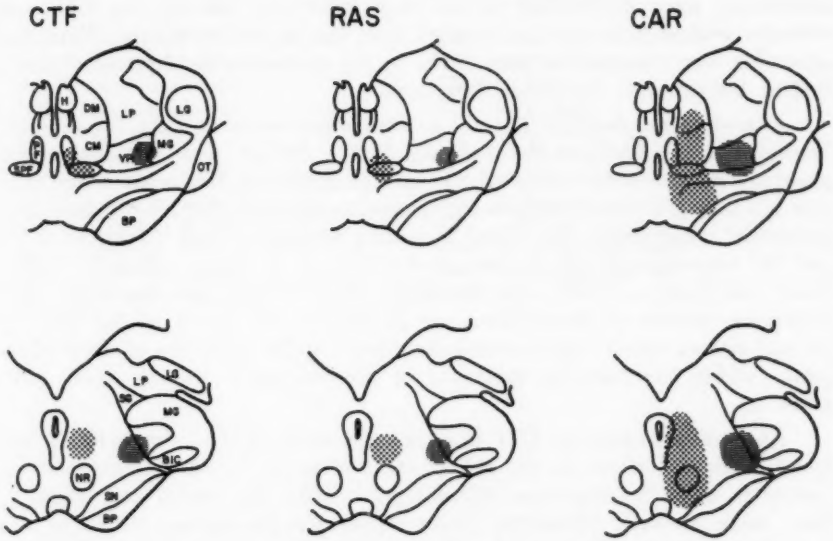


Fig. 10. Comparisons of the anatomical disposition (stippling) of the central tegmental fasciculus (CTF), reticular arousal system (RAS), and collateral afferent reticular projection (CAR) at the midbrain and posterior thalamus. There is a close correlation between the CTF and RAS; the CAR projection involves a larger proportion of the brainstem at both levels. The anterolateral projection is indicated by hatching in the CTF figure, it corresponds with the lateral region (hatched) giving arousal with stimulation in the RAS figure (the medial lemniscus is not active in arousal). The hatched area in the CAR figure embraces both the medial lemniscus and the anterolateral system activated by sciatic stimulation. (Based on Starzl *et al.*, 1951 a, b; and Kerr, 1961.)

et al., 1953). Both systems exhibit convergence of afferents from diverse sources (Amassian and DeVito, 1954, for CAR).

If one compares the reticular arousal and CTF systems at two typical levels in the brainstem (Fig. 10), it will be seen that the arousal system (RAS), as defined by Starzl *et al.* (1951a) is for all practical purposes identical with the central tegmental fasciculus (CTF). Our interpretation of some of the nuclear configuration of the thalamus differs from that of Starzl *et al.* but this does not affect the argument. So far as evoked potentials are concerned, the collateral afferent projection (CAR) of the reticular system (Starzl *et al.*, 1951b) is somewhat similar to that of the CTF and RAS, but the reticular evoked responses are also recorded from a more ventral extent of the midbrain embracing the red nucleus and the subthalamus. This region ventral to the CTF is a site from which it is possible to record long latency evoked responses which exhibit sensitivity to general anaesthetics, being depressed in amplitude at subanaesthetic dosages (French *et al.*, 1953; Haugen and Melzack, 1957). This sensitivity to

anaesthesia must be stressed as the main difference between the CTF and reticular evoked potentials as recorded from the upper brainstem. Note that arousal is also obtained by stimulation of the anterolateral ("spino-thalamic") system, but not from the medial lemniscus.

It now seems doubtful if one can very often stimulate only the reticular arousal system (RAS) at the midbrain level. Rather, such stimulation will generally involve direct fibres of the central tegmental fasciculus which contain not only the spinal components demonstrated here, but also at least two trigeminal components, the dorsal secondary trigeminal tract (Torvick, 1957) and the trigemino-reticulo-thalamic path (Kerr *et al.*, 1955). Russell (1954), Nauta and Kuypers (1958), and Brodal and Rossi (1955) have also shown that ascending systems of direct fibres are present in the region of the reticular arousal system which was formerly considered to be a system of short chain relays within the reticular substance of the brainstem (Starzl *et al.*, 1951, a and b).

An attempt to miss the CTF in stimulation of the RAS at the midbrain and tectal levels will often run the risk of stimulating the brachium conjunctivum ventral to it, or the trigeminal afferent paths which lie ventral and lateral to this. More medially stimulation might involve the mammillary peduncle in a ventral situation, or the central grey more dorsally. In short, stimulation of the reticular arousal system is seemingly a difficult procedure not often likely to be achieved.

REFERENCES.

Amassian, V. E., and DeVito, R. V. (1954): J. Neurophysiol., 17, p. 575.
Anderson, F. D., and Berry, C. M. (1959): J. comp. Neurol., 111, p. 195.
Brodal, A., and Rossi, G. F. (1955): A.M.A. Arch. Neurol. and Psychiat., 74, p. 68.
Clezy, J. K. A., Dennis, B. J., and Kerr, D. I. B. (1961): Austral. J. exp. Biol., 39, p. 19.
Carpenter, M. B. (1957): J. Anat., 91, p. 82.
Dennis, B. J., and Kerr, D. I. B. (1961): Austral. J. exp. Biol., 39, p. 29.
French, J. D., Verzeano, M., and Magoun, H. W. (1953): A.M.A. Arch. Neurol and Psychiat., 69, p. 519.
Haugen, F. P., and Melzack, R. (1957): Anaesthesiol., 18, p. 183.
Kerr, D. I. B.: To be published.
Kerr, D. I. B., Haugen, F. P., and Melzack, R. (1955): Amer. J. Physiol., 183, p. 253.
Lewandowsky, M. (1904): In O. Vogt. Neurobiol. Arbeiten II. Serie, Bd. 1.2, p. 63.
Magoun, H. W. (1952): A.M.A. Arch. Neurol. Psychiat., 67, p. 145.
McMasters, R. E., and Russell, G. V. (1958): J. comp. Neurol., 110, p. 205.
Mehler, W. R. (1957): Anat. Rec., 127, p. 332.
Nauta, W. J. H., and Kuypers, H. G. J. M. (1958): In "Reticular Formation of the Brain". Henry Ford Hospital. International Symposium (H. H. Jasper, Ed.) Little, Brown. Boston. p. 3.
Russell, G. V. (1954): J. comp. Neurol., 101, p. 237.
Starzl, T. E., Taylor, C. W., and Magoun, H. W. (1951a): J. Neurophysiol., 14, p. 461.
Starzl, T. E., Taylor, C. W., and Magoun, H. W. (1951b): *Ibid.*, 14, p. 479.
Torvick, A. (1957): Amer. J. Anat., 100, p. 1.

ABBREVIATIONS FOR ALL FIGURES

ALC	anterolateral column pathway (anterolateral system)
BC	brachium conjunctivum
BIC	brachium of inferior colliculus
BP	basis pedunculi
CG	central grey
CM	n. centre median
CTF	central tegmental fasciculus
DM	n. dorsalis medialis
H	habenula
LG	lateral geniculate n.
LP	n. lateralis posterior
MG	medial geniculate n.
ML	medial lemniscus
NR	n. ruber
OT	optic tract
P	pons, brachium pontis
PF	n. parafascicularis
PG	n. parageniculatus
SG	n. supragenicultatus
SN	substantia nigra
SPF	n. subparafascicularis
VP	n. ventralis posterior

THE CLOTTING OF THE BLOOD OF AN AMPHIBIAN, *BUFO MARINUS* LINN.

1. PROTHROMBIN-THROMBIN AND "FIBRINOGEN-FIBRIN" STAGES

by EARLE HACKETT AND REX LEPAGE[1]

(From the Medical Research Division, Institute of Medical and Veterinary Science, Adelaide, South Australia).

(*Accepted for publication 6th January, 1961.*)

SUMMARY.

The blood clotting mechanism of the toad *Bufo marinus* was investigated *in vitro* using techniques commonly employed in the study of the prothrombin-thrombin and fibrinogen-fibrin stages of human blood coagulation.

Clotting of the toad's blood depends upon the presence of calcium ions. The optimum pH is 7·8 and the optimum temperature 35°-40° C. The clot is jelly-like rather than fibrin-like and is formed by the coagulation of a "fibrinogen". Clotted blood becomes fluid again in 2-4 hours through the action of a serum lysin. A prothrombin which is converted quantitatively to a thrombin was demonstrated. The prothrombin is converted by a toad blood leucocyte thromboplastin which is as active as some toad tissue extracts. One-stage techniques using intrinsic (i.e. blood) sources of thromboplastin revealed no plasma factors similar to human factor V or to anti-haemophilic globulin. Some of the principles to be followed in the investigation of the blood coagulation mechanism of an animal such as the toad are discussed.

INTRODUCTION.

Any broad understanding of the blood coagulation system of mammals must include an insight into the biological evolution of this apparently intricate plasma mechanism. An investigation of the blood of a lower vertebrate might throw some light upon it. Accordingly, we have studied the clotting *in vitro* of the blood of an amphibian, the toad *Bufo marinus* Linn., using present knowledge of the human blood coagulation system as a guide.

This animal co-exists with sugar-cane plantations in Central and South America, the West Indies, the Indo-Pacific region, and Australia. When we refer in these papers to "toad blood", we mean the blood of *Bufo marinus* only.

MATERIALS AND METHODS.

Toads. The animals were taken by a professional collector[2] in Queensland and sent by air freight to Adelaide. They survived well in a moist, unheated, shaded vivarium in the

[1] Present address: Department of Pathology, University of Melbourne.
[2] H. J. Hohnke, Biological Service, P.O. Box 193, Brisbane.

animal house of this Institute. The mean weights of a typical consignment were: males (79 toads), mean wt. 134 gm., S.D. 23 gm.; females (45 toads) mean wt. 197 gm., S.D. 56 gm.

Bleeding technique. The method of Hsiao, quoted by Cameron (1950) was modified as follows. The animal was cleanly pithed and pinned supine with its forelimbs widespread. The pectoral skin was opened and the sternum cut off with sharp scissors. The cartilage uniting the clavicles and coracoid bones was cut through in the mid-line, revealing the pericardial sac which was then opened to fully expose the still beating heart and the great arteries. Care was taken to avoid unnecessary crushing or tearing of tissues during the exposure. Three ligatures were placed, but not tightened, two on the left and one on the right main branch of the bulbus arteriosus. A very light spring clamp with flat jaws 6·0 mm. broad (Dieffenbach's small artery forceps with its spring weakened by grinding) was put on the bulbus itself, temporarily stopping all flow. A 10·0 cm. length of polyethylene tube, internal diameter 1·0 mm., was inserted towards the heart through a small incision into the left branch of the bulbus between the ligatures. The three ligatures were then tied so that all blood flowing from the heart would be diverted into the cannula, and the clamp was released. Blood from the cannula was collected in an appropriate vessel (e.g. plastic or glass, siliconed or waxed, etc.), with anticoagulant if required. 5·0 to 8·0 ml. of blood could be obtained quickly from each toad by this method.

When liquid citrate or oxalate anticoagulants were used in standard proportions the animals were bled directly into these solutions in graduated tubes on the assumption (based on experience) that a toad of given heart size would yield a certain volume of blood, more anticoagulant being added immediately if the amount of blood proved unexpectedly high.

Anticoagulants. 0·1 M sodium oxalate or 0·12 M sodium citrate was used in the proportion of 1 part anticoagulant to 9 parts blood. Dipotassium ethylenediamine tetra-acetic acid (sequestrene) was used at a strength of 2 mgm./ml. blood. Heparin (Organon) was used at 200 units/ml. blood; this may have been unnecessarily strong.

Physiological saline (toad). 0·7 p.c. sodium chloride solution was used.

Calcium chloride. 0·025 M calcium chloride was the optimum strength for recalcified clotting of oxalated or citrated toad blood.

Temperature. Experiments were carried out at 37° C. except where otherwise stated.

pH. pH was determined with a Jones pH meter and a glass-electrode. The optimum pH for recalcified clotting of toad blood was 7·8.

Plasma. Plasma rich in leucocytes was prepared by centrifuging anticoagulated blood at 2,300 G for 10 seconds. This sedimented the red cells, leaving many leucocytes still suspended. Plasma free of leucocytes was prepared by recentrifuging leucocyte-rich plasma at 800 G for 10 minutes: this gently sedimented the leucocytes in a state in which they could be saved and resuspended should this be required. The supernatant plasma was then centrifuged again at 2,300 G for 10 minutes. This gave a plasma free of visible leucocytes when examined under the phase contrast microscope. A veronal buffer at pH 7·8 was used when buffered dilution of toad plasma was necessary.

Leucocyte suspensions. Leucocyte-rich plasma (see above) was centrifuged at 800 G for 10 minutes, the supernatant was removed and the sediment gently resuspended in 0·7 p.c. sodium chloride solution to 1/3rd of the original volume. This centrifugation and resuspension in saline was repeated twice to free the cells of any residual prothrombin or other plasma blood clotting factors. Waxed glassware and pipettes were used throughout if glass-contact was to be avoided. The cells of this leucocyte-rich plasma or leucocyte suspension contained about 70 p.c. spindle cells which settled more slowly than the other types of toad white cells (cf. Tait and Green, 1926). We found no blood platelets in the toad.

Serum. Blood was collected into plain glass tubes to which 1 gm. of crushed glass powder had been added to ensure rapid and complete clotting. After separation, the serum was pipetted off and kept, if necessary, for a few days at 4° C. or stored for longer periods at −20° C.

Other reagents and techniques.

The following reagents and techniques were prepared or carried out according to the recommended methods for human blood described or referred to in the technical appendices of *Human Blood Coagulation and its Disorders* by Biggs and Macfarlane (1957): Acetone-dried toad-brain extract; thromboplastic tissue-extracts; whole blood coagulation times; calcium clotting times (which we refer to here as recalcified clotting times); silicone treatment of glass; alumina and barium sulphate adsorption of plasma or serum; thrombin generation tests; and barium sulphate/sodium citrate adsorption and elution of prothrombin. Any special points occasioned by the use of toad blood rather than human blood are mentioned in the rest of the paper as they arise.

RESULTS.

Effect of anticoagulants.

The following anticoagulants were effective in preventing toad blood coagulation *in vitro*: sodium citrate, sodium oxalate, ammonium and potassium oxalate mixture, dipotassium ethylenediamine tetra-acetic acid (sequestrene), and heparin. Citrated or oxalated plasma was clotted by the addition of excess calcium chloride. Citrated, oxalated, or heparinised blood was clotted by the addition of excess bovine thrombin. Toad serum could not be re-clotted and had a lower specific gravity than plasma.

By analogy with the mechanisms believed to underlie the effects of these anticoagulants upon the blood of warm-blooded vertebrates we have inferred that free calcium ions are necessary for one or more essential stages in the coagulation of toad blood and that a fibrinogen-like substance in toad plasma is clotted by a thrombin.

Whole-blood coagulation time.

Untreated freshly-drawn toad blood in 1·0 cm. diameter test tubes clotted at various rates according to the nature of the surface in the tube (Table 1). The firmness of the clot and the rate of coagulation were proportional to one another (the firmer, the faster), and were enhanced by the presence of siliceous surfaces or particles. However, there was an apparent lack of uniformity in the effects of some "non-wettable" surfaces on the coagulability of toad blood. In the next paper in this series an investigation of these effects is described and an interpretation offered.

The toad blood clot did not have the fibrous structure of a mammalian blood clot and the term "fibrin" is not appropriately descriptive, but we have used it because of the analogy. We found it impossible to wash and press a toad plasma clot free of serum because of its soft jelly-like nature. It could

TABLE 1.

Plain coagulation times of toad blood (Lee and White method).

Experiment No.	Temp.	Toad No.	Surface in test tube	Clotting begins (seconds)	Clotting complete (seconds)
1	24° C.	1 2 3	Acid-washed plain glass tube	100 120 90	405 560 678
2	24° C.	1 2 3	Siliconed plain glass tube	No visible clotting within 2½ hours	
3	24° C.	4 5 6	Paraffin-waxed glass tube	No visible clotting within 24 hours	
4	24° C.	7 8	Paraffin-waxed glass tube with 5 p.c. by vol. acid-washed fine glass powder added to blood	Almost immediately	90 150
5	24° C.	7 8	Paraffin-waxed glass tube with 5 p.c. by vol. siliconed fine glass powder added to blood.	165 180	390 360
6	22° C.	9 10	Paraffin-waxed glass with 5 p.c. by vol. kaolin powder added to blood	Immediately	75 68
7	22° C.	9 10	Paraffin-waxed glass with 5 p.c. by vol. kieselguhr powder added to blood	Immediately	80 92
8	22° C.	9 10	Celluloid tube	No visible clot within 1 hour	
9	22° C.	9 10	Perspex tube	No visible clot within 1 hour	
10	22° C.	9 10	Nylon tube	300 300	505 770
11	22° C.	9 10	Polyethylene tube	No visible clot within 1 hour	

not be obtained in the tough thready form of a mammalian blood clot, even when clotted with added silicaceous powders or bovine thrombin. We were therefore unable to estimate the "fibrinogen" level of samples of toad plasma by the usual method of measuring the protein in a washed plasma clot.

Lysis of the clot.

A clot formed by toad blood *in vitro* usually redissolved in its serum in 2-4 hours and the red cells which had been trapped in it sedimented without haemolysis. Experiments in which fresh clots were transferred to different serum samples showed the presence of a storage-labile serum fibrinolysin active both at 37° C. and bench temperature.

Such lytic activity is greater than that observed in the sera of humans unless they have been subjected to trauma or exceptional physical or psychological stress which tend to stimulate fibrinolysis. We considered the possibility that the clot lysis in the toads might have been stimulated, or increased, by our manner of pithing them before bleeding. As the bleeding of toads for coagulation studies by percutaneous venipuncture is not possible, a number of animals was anaesthetised with pentobarbitone, handled very gently and bled in the usual way but without pithing. The bloods so obtained showed no departure from the whole-blood coagulation times or recalcified clotting times which we had already observed and all clots underwent lysis in 2-4 hours.

Effects of temperature and leucocytes on recalcified clotting times of citrated blood.

The effects of temperatures between 5° C. and 47° C. on the recalcified clotting time ("calcium clotting time") of pooled citrated leucocyte-free and leucocyte-rich plasma are shown in Fig. 1. It was clear that the presence of leucocytes in ordinary numbers definitely accelerated plasma clotting but our method of preparing "leucocyte-free plasma" may not have removed every single cell fragment so that we cannot presume to have observed the effect of a complete absence of leucocytes on the recalcified clotting time.

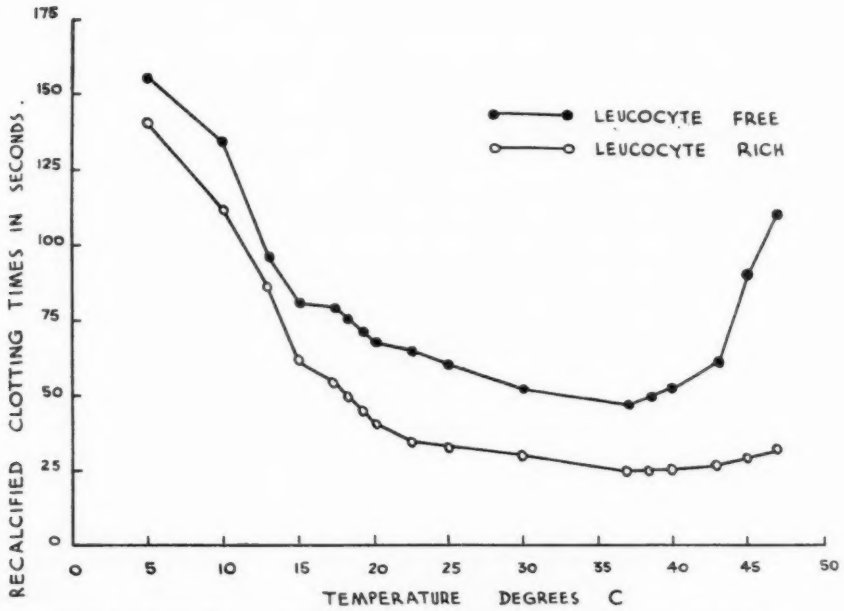


Fig. 1. Recalcified clotting times at temperatures ranging from 5° C. to 47° C.

The smooth shape of the graph gave no indication of an agent inactive at low temperatures suddenly coming into play as the temperature was raised from 10° to 40° C. The optimum temperature for the recalcified clotting of toad blood was 35°-40° C. At lower temperatures (5°-10° C.) the recalcified clotting time was faster than that of human plasma at the same temperature.

Recalcified clotting times with added tissue or leucocyte preparations.

Recalcified clotting times of oxalated or citrated leucocyte-free toad plasma were shorter when a homologous tissue extract or acetone-dried brain powder was added (Table 2). The same is, of course, found with human plasma. However, a saline suspension of toad blood leucocytes was as active an accelerator of the recalcified clotting time as acetone-dried brain powder or as some tissue extracts. Cell-free washings of a leucocyte suspension were also effective.

TABLE 2.

Recalcified clotting times of citrated cell-free toad plasma with various added thromboplastic substances.

Substance added in unit volume to pooled cell-free toad plasma	Recalcified clotting times (seconds)	
	16° C.	37° C.
Saline (control)	153	77
Saline extract of toad brain	26	15
Acetone-dried toad brain suspended in saline	53	22
Saline extract of toad spleen	49	24
Toad leucocytes suspended in saline	88	23
Cell-free saline washings of toad leucocytes	91	28

This marked "thromboplastic" activity of prepared toad leucocytes or their washings differed from the known effect of mammalian blood platelets or leucocytes on centrifuged plasma where they have little influence on the one-stage recalcified clotting time. "Complete" thromboplastic agents such as homologous tissue extracts or acetone-dried brain powder have a marked accelerating effect on recalcified mammalian plasma. ("Completeness" is indicated by the speed with which a thromboplastin can convert prothrombin to thrombin and by the extent of its independence of serum or plasma co-factors.) It appeared, then, that prepared toad leucocytes, or some cell-types among them, were able to act as a relatively "complete" blood thromboplastin, comparable in activity to toad tissue thromboplastin. Further investigation of toad leucocytes and their thromboplastic activity is the subject of further papers (Hackett and LePage, 1961b, 1961c).

Adsorption of plasma with alumina.

Adsorption of citrated toad plasma with alumina, or of oxalated plasma with barium sulphate, did not affect the "fibrinogen" level as judged by the quality of the clot, but it markedly lengthened the one-stage recalcified clotting time with added tissue extracts or leucocyte suspensions. The adsorption was progressive, increasing the clotting time in proportion to the time for which the plasma and the alumina were in contact.

Graphs plotted from two-stage tests upon recalcified mixtures of alumina-adsorbed leucocyte-free plasma and normal unadsorbed plasma showed that a thrombin-like reagent was generated in direct proportion to the amount of whole unadsorbed plasma in such mixtures. The amount of thrombin generated was measured arbitrarily by reference to a dilution curve of the activity of a preparation of bovine thrombin on toad plasma. Bovine thrombin was just half as active in clotting toad plasma as it was with human plasma.

We concluded that alumina adsorbed a soluble non-cellular precursor in toad plasma which was converted quantitatively to a thrombin. This precursor, again by analogy with the mammalian coagulation system, was called "prothrombin".

Further evidence for the quantitative conversion of prothrombin to thrombin in toad plasma.

We adsorbed oxalated cell-free toad plasma with barium sulphate which was then washed in water and eluted with trisodium citrate solution. The eluate was dialysed against citrate-saline and yielded a solution which could fully restore to normal the recalcified clotting time of alumina-adsorbed plasma with added toad tissue extract or leucocytes. When the eluate was used at full concentration the recalcified times were made shorter than those of normal plasma. When these times were related to a bovine thrombin dilution curve, as in the previous experiment, they indicated that five times the normal amount of toad thrombin had been generated, and consequently it seemed that the precursor, toad plasma prothrombin, must have been concentrated in the eluate to this extent. This was further evidence of a quantitative part played by a prothrombin in the toad's blood clotting system.

No plasma co-factors of prothrombin observed by one-stage testing in glass.

Ageing of uncontaminated toad plasma in glass for periods of several days at room temperature steadily increased its recalcified clotting time with tissue extracts or leucocytes. This falling-off was not counteracted by the addition of alumina-adsorbed fresh plasma or by unadsorbed fresh plasma diluted 1/20, and the aged plasma had no anticoagulant effect upon fresh unadsorbed plasma.

This indicated that the only change in ageing plasma was a diminution of its prothrombin. If there were any other soluble toad plasma coagulation factor

accelerating the conversion of prothrombin to thrombin and demonstrable in glass by a one-stage technique using intrinsic sources of thromboplastin it must have been more stable on storage than prothrombin itself, adsorbable with alumina, and not an enzyme-like agent able to withstand dilution to 1/20. It would therefore have been indistinguishable from prothrombin itself, and unlike human factor V or anti-haemophilic globulin.

An investigation of *serum* coagulation factors in the toad is described in the next paper in this series

DISCUSSION.

The optimum temperature for blood coagulation in *Bufo marinus*, a poikilothermic animal, was approximately 37° C. This might be regarded as an indication of a preadaptation to the homoiothermic life at this temperature eventually realised by the birds and mammals. It is indeed possible that 35°-40° C. is the optimum temperature for a wide complex of metabolic reactions evolved at lower temperatures, but it must be remembered that *Bufo marinus* spends its active life in warm tropical sugar-cane plantations where its body temperature may remain for long periods at much the same level as that of a "warm-blooded" animal, and this could have resulted in the development of mechanisms which work best at such temperatures.

The most important difference between the coagulation of toad blood and of human blood which we report in this paper is that a preparation of toad blood leucocytes was a source of an intrinsic thromboplastin which was almost as "complete" as many toad tissue extracts, and that toad plasma had neither a factor V-like plasma accelerator of prothrombin conversion, nor an anti-haemophilic-globulin-like co-factor of intrinsic thromboplastin activity.

It might be thought that the distribution of blood coagulation factors in various vertebrates ought to be investigated simply by making experimental clotting mixtures of human coagulation factors (which have been well characterised) with those of other animals and noting the effects of the substitutions. This presumes that the human factors have the general interchangeable nature of pure chemical reagents. Such a method probably has its pitfalls. However, assumptions, techniques and even plasma or serum derivatives used in human blood coagulation experiments have been successfully transferred to the study of blood clotting in higher vertebrates, for example, in the horse (Barkhan, Tomlin and Archer, 1957), in some marsupials (Fantl and Ward, 1957) and in a bird (Wartelle, 1957). But experiments which involve mixing bloods, plasmas, or sera, of different species are open to the objection that ensuing reactions are likely to be influenced by species specificities of proteins, by antibody effects, and by unknown optimum requirements of the enzymes, substrates and plasma proteins which compose the "clotting factors". For instance, thrombin, which is a well characterised clotting factor and very general in its

action on vertebrate blood, is species specific in its optimum activity even among mammals (Stormorken, 1957).

In the course of our experiments described here, we made many premature attempts to substitute toad blood preparations in human clotting mixtures and *vice versa*. The results were irregular. Occasionally some interaction took place, but it was usually followed by rapid deceleration and lack of further activity.

This initial investigation of the toad's blood coagulation system has therefore been confined to the use of substances deriving from the toad itself, and as far as possible taken only from its blood. Our experience suggests that the avoidance of interspecific mixtures whenever possible should be a principle in any preliminary experimental study of the blood coagulation of a lower vertebrate.

Acknowledgments. We wish to acknowledge the valuable technical help and advice of Mr. John Marshall who, with Miss Maureen Dangerfield, performed some thousands of coagulation tests with us.

REFERENCES.

Barkhan, P., Tomlin, S. C., and Archer, R. K. (1957): J. comp. Path., 67, p. 358.
Biggs, R., and Macfarlane, R. G. (1957): *Human Blood Coagulation and its Disorders.* 2nd ed. Blackwell Scientific Publications, Oxford.
Cameron, G. (1950): *Tissue Culture Technique.* 2nd ed., p. 51. Academic Press, New York.
Fantl, P., and Ward, H. A. (1957): Austral. J. exp. Biol., 35, p. 209.
Hackett, E., and LePage, R. (1961b): *Ibid.*, 39, p. 67.
Hackett, E., and LePage, R. (1961c): In preparation.
Stormorken, H. (1957): Acta. Physiol. Scand., 40, p. 167.
Tait, J., and Green, F. (1926): Quart. J. Exp. Physiol., 16, p. 141.
Wartelle, O. (1957): Rev. d'Hématologie, 12, p. 351.

THE CLOTTING OF THE BLOOD OF AN AMPHIBIAN, *BUFO MARINUS* LINN.

2. BLOOD THROMBOPLASTIC ACTIVITY

by EARLE HACKETT AND REX LEPAGE[1]

(From the Medical Research Division, Institute of Medical and Veterinary Science, Adelaide, South Australia).

(*Accepted for publication 6th January, 1961.*)

SUMMARY.

Investigation of the intrinsic thromboplastic activity of the blood of the toad *Bufo marinus* Linn. shows that this arises largely from the blood leucocytes. More prothrombin appears to be consumed in leucocyte-rich than in leucocyte-free mixtures. A spreading change which occurs in the toad thrombocyte (a spindle-shaped leucocyte) on contact with certain surfaces is morphologically analogous to the "viscous metamorphosis" which occurs in the human platelet. However, the change in the thrombocyte is not inhibited by decalcifying anticoagulants or heparin and takes place on plain glass, siliconed glass and perspex; somewhat less rapidly on cellulose acetate; and not at all on paraffin wax or polyethylene.

The thromoplastic activity of plasma-free leucocytes appears after contact with glass, siliconed glass, or perspex, but not after contact with paraffin wax, and so the development of the leucocyte thromboplastic activity after surface contact runs parallel to the morphological change observed in the thrombocytes.

A non-cellular plasma co-thromboplastic factor activated by contact with glass (but not siliconed glass) is also found. It does not depend on calcium ions for its activation or for its activity. It reacts with the thromboplastin released by glass-treated or siliconed-glass-treated leucocytes to convert it to a still more active thromboplastin, this conversion itself being accelerated by the presence of a glass (but not by a siliconed glass) surface.

An alumina-adsorbable serum accelerator appears after clotting and a serum anticoagulant is also found.

The plasma co-thromboplastic contact factor has properties comparable to those of human Hageman factor, and the serum accelerator to those of human Christmas factor.

The findings are discusseed in relation to a possible stage in the evolution of the human blood coagulation system.

A scheme for the coagulation mechanism of the blood of *Bufo marinus* is presented (Fig. 1).

INTRODUCTION.

We have shown in the first paper in this series (Hackett and LePage, 1961a) that free calcium ions, "fibrinogen", prothrombin and leucocytes are plasma coagulation factors which can be demonstrated by a one-stage recalcification

[1] Present address: Department of Pathology, University of Melbourne.

technique in the blood of the toad *Bufo marinus* Linn. The toad's plasma lacks factors corresponding to the human factor V or anti-haemophilic globulin. The toad leucocytes seem to provide a notably "complete" thromboplastin, being as active in this respect as some tissue extracts. Such "completeness" can be contrasted with the relatively "incomplete" contribution made to intrinsic (i.e. blood) thromboplastin generation by human platelets.

The present paper describes further investigations of intrinsic thromboplastic activity in the blood of *Bufo marinus*.

MATERIALS AND METHODS.

General. The methods of obtaining toads, of bleeding them and of preparing plasma, serum and cell suspensions were the same as in the previous paper. Acetone-dried toad brain extract, silicone treatment of glass, alumina adsorption of plasma, recalcified clotting times (or "calcium clotting times"), and prothrombin consumption tests were prepared or carried out according to the recommended methods for human blood described or referred to in the technical appendices of *Human Blood Coagulation and its Disorders* by Biggs and Macfarlane (1957). Any special points occasioned by the use of toad, rather than human, blood are mentioned below as they arise.

Concentrated leucocyte suspensions. Citrated blood was collected in paraffin-waxed glass tubes and centrifuged at 2,300 G for 10 seconds. The supernatant leucocyte-rich plasma was transferred, using a waxed pipette, to similar tubes, and re-centrifuged at the same speed for 15 minutes. The supernatant leucocyte-free plasma was removed and the sedimented cells at the bottom of the tube were washed three times by gently resuspending in fresh 0·7 p.c. saline and re-centrifuging at 2,300 G for 5 minutes. The final cell sediment was resuspended in a volume of saline calculated to give whatever experimental concentration of leucocytes might be required (e.g. as shown in Table 2) relative to that of the original plasma from which they had derived, assuming no loss in handling.

Supernatant saline extract of leucocytes. The third saline supernatant prepared as above from the washed leucocytes was used. For certain experiments, specifically indicated in Table 3, the leucocyte button was deliberately mashed with a wooden probe before each centrifuging.

Thromboplastic toad tissue extracts. (*Extrinsic thromboplastin.*) Tissues were dissected from freshly-pithed toads, cleared of extraneous blood and vessels, and rinsed six times in 0·7 p.c. saline. The wet tissue was triturated in ten times its volume of saline using sand (May and Baker, medium fine, acid-washed) with a glass pestle and mortar. The liquid was then decanted from the mortar and centrifuged at 2,300 G for 5 minutes. The supernatant fluid was used as the tissue-thromboplastin solution or suspension and it was always freshly prepared before use.

Phase-contrast microscopy. A Leitz Dialux microscope was used with phase-contrast attachments and a temperature-controlled stage.

RESULTS.

Effect of various tissue preparations, leucocytes and leucocyte extracts, on the recalcified clotting time.

The recalcified clotting times of cell-free toad plasmas were greatly accelerated by the addition of saline extracts of toad tissues or of saline-washed toad leucocyte suspensions. The activity of various preparations added to pooled

TABLE 1.

Recalcified clotting times of cell-free citrated toad plasma with various added thromboplastic substances.

Substances added in unit volume to pooled cell-free toad plasma	Recalcified clotting times (seconds)	
	16° C.	37° C.
Saline (control)	153	77
Saline suspension of acetone-dried toad brain	53	22
Saline extract of wet toad brain	26	15
Saline extract of wet toad lung	26	9
Saline extract of wet toad kidney	36	14
Saline extract of wet toad skin	46	18
Saline extract of wet toad muscle	48	18
Saline extract of wet toad heart	39	18
Saline extract of wet toad liver	63	28
Saline extract of wet toad spleen	49	24
Toad leucocytes suspended in saline	88	23
Cell-free saline washings of toad leucocytes	91	28

plasma is shown in the typical experiment summarised in Table 1. In most cases the reaction was about twice as fast at 37° C. as at 16° C., and the same relative order of thromboplastic activity among the extracts was observed whenever this experiment was repeated.

Acetone-dried toad brain powder proved to be a stable thromboplastic tissue preparation, the brains of 50 toads yielding 0·4 gm. of dry powder. This preparation did not accelerate a recalcified clotting time to the same degree as did a wet brain emulsion.

Washed leucocyte suspensions were not effective in restoring the recalcified clotting times of samples of alumina-adsorbed plasma. We therefore presumed that the cells contributed thromboplastin and not prothrombin.

We measured the rate of prothrombin consumption in recalcified leucocyte-rich and cell-free plasma using a two-stage technique with alumina-adsorbed toad plasma as a substrate. More prothrombin seemed to be consumed (because less prothrombin-like activity remained) and the maximum rate of consumption was reached sooner in leucocyte-rich plasma. The addition of acetone-dried toad brain powder to cell-free plasma brought its prothrombin-consumption up to the same level as that of leucocyte-rich plasma. This interpretation of the prothrombin consumption tests assumes that toad leucocytes were not a source or part-source of any heparin-like anticoagulant. We report later in this paper, however, that an anti-thrombin was demonstrable in fresh toad serum, and the possibility remains that leucocytes play a part in its production. If they do, the apparent greater utilisation of prothrombin in the leucocyte-rich plasma might have been due to the action of this anticoagulant, and so the result cannot be taken unequivocally to support the other evidence that the activity of leucocytes is thromboplastic.

TABLE 2.

Thromboplastic effect in citrated toad plasma of various concentrations of toad leucocytes.

Concentrations of leucocyte suspensions in saline (relative to normal in blood) added in unit volume to toad plasma	Recalcified clotting time at 37° C. (seconds)
Nil (control)	61
1	28
3	25
10	23
25	22
50	21
75	22
100	23

The effect of using leucocytes at various concentrations is shown in Table 2. As these suspensions had been prepared in waxed tubes they were subjected to optimum contact with siliconed glass to activate the thrombocytes (see later experiments on the effects of glass contact). A concentration of ten times their normal numbers gave optimum leucocyte thromboplastic activity, with little or no enhancement at concentrations up to 100 times normal. The difference between a ten times concentration and one of three times, which we found convenient to use in all our general experiments, was small.

Supernatant cell-free saline washings of leucocyte suspensions centrifuged in glass at 2,300 G for 10 minutes were effective accelerators of the recalcified clotting times of cell-free plasma but not as active as the suspensions themselves. Multiple saline washings of a leucocyte button with vigorous resus-

TABLE 3.

Thromboplastic effects of centrifuged saline extracts of toad leucocytes added to citrated cell-free toad plasmas.

	Solution added in unit volume to plasma	Recalcified clotting times at 37° C.(seconds)
Experiment 1	Saline (control)	80
Broken leucocytes	1st supernatant	25
Cell button mashed with wooden probe before each resuspension	5th supernatant	44
	7th supernatant	74
Experiment 2	Saline (control)	79
Broken leucocytes	Undiluted	33
Effect of saline dilution upon 3rd supernatant from experiment 1	Diluted 1 in 2	36
	Diluted 1 in 4	44
	Diluted 1 in 8	46
	Diluted 1 in 16	54
	Diluted 1 in 32	76
Experiment 3	Saline (control)	50
Whole leucocytes	1st supernatant	31
Cell button resuspended without mashing	5th supernatant	32
	10th supernatant	36
	30th supernatant	45

pension each time in saline to one-third of the original plasma volume from which the cells derived gave a consecutive series of active saline supernatant extracts in which some thromboplastic activity was still detectable up to the seventh wash in one experiment where the leucocyte button was energetically broken with a wooden probe between each wash, and to the thirtieth in another where the cells were simply sedimented without any manipulation intended to break them (Table 3).

Washed toad erythrocytes in 1 p.c. suspension in saline showed a barely detectable thromboplastic activity. This effect could have been ascribed to a few contaminating leucocytes.

Toad thrombocytes.

We understand the term "thrombocyte" to mean a spindle cell of a type found in birds, reptiles, amphibians and fishes. When it comes in contact with a glass surface this cell undergoes a spreading change in shape, similar to the "viscous metamorphosis" of the mammalian blood platelet. We do not use the term "thrombocyte" for the latter.

Microscopic examination showed that the leucocyte suspension prepared by centrifuging citrated toad blood at 2,300 G for 10 secs. was in fact a thrombocyte concentrate, 70 p.c. of the cells being thrombocytes, compared with an average proportion of 50 p.c. thrombocytes which we observed in differential leucocyte counts on stained films of toad blood (Hackett and LePage, 1961c). We found no blood platelets in *Bufo marinus*.

We investigated the conditions under which the surface-spreading "viscous metamorphic" change took place in the toad thrombocytes, using a phase-contrast microscope, with these results: the change was hastened by warming to 37° C., but proceeded nearly as rapidly at room temperature (27° C.); it was unaffected by the presence of sodium citrate, sodium oxalate, dipotassium ethylenediamine tetra-acetic acid ("sequestrene"), or heparin.

This observation that various decalcifying anticoagulants did not prevent the contact-change of toad thrombocytes on glass is not in accord with the findings of Pringle and Tait (1910, 1911) on frog's blood; neither is the same found with human platelets, which will not undergo "viscous metamorphosis" in plasma which lacks calcium ions (Sharp, 1958).

The cells changed as rapidly on siliconed as on plain glass, but showed no alteration in shape after 24 hours on paraffin wax. These observations on the effect of anticoagulants, glass, siliconed glass, and wax were repeated satisfactorily many times. Further experiment showed that celluloid and polyethylene surfaces were almost as non-reactive as paraffin wax, that cellophane and nylon surfaces delayed the change somewhat but did not prevent it, and that perspex was as active in promoting it as glass or siliconed glass (Table 4).

The spreading behaviour of toad thrombocytes on these various kinds of surface generally ran parallel with the effect of the surface on the clotting time

TABLE 4.

Viscous metamorphosis of toad thrombocytes. Times for the change to take place in citrated toad plasma on various transparent surfaces at room temperature.

Surfaces	Change begins (minutes)	Change complete (minutes)
Acid-washed glass	3	6-7
Siliconed glass	3	5
Paraffin wax	No change	
"Cellophane"	8	15
Celluloid	No change	
Polyethylene	No change	
Nylon	7-8	20
Perspex	3	5-6

The cells on the surfaces marked "no change" were observed for 2 hours in this experiment but cells were frequently observed unchanged after 24 hours on wax in other experiments.

of whole toad blood. Clotting was rapid when the surface caused fast spreading. When there was no spreading clotting was delayed or absent, except for an anomaly in the case of siliconed glass and of perspex, on both of which the thrombocytes changed readily. We had previously shown (Hackett and LePage, 1961a, Table 1) that the use of a siliconed or perspex test tube greatly delayed toad blood coagulation and that siliconed glass powder added to toad blood in a waxed tube resulted in a much longer clotting time than when plain glass powder was used. This conflicted with the visual evidence that toad thrombocytes underwent "viscous metamorphosis" as quickly and completely on siliconed glass as on plain glass. It therefore seemed that while "viscous metamorphosis" of the thrombocytes might play a part in the clotting of toad blood there was evidently some other surface-sensitive factor or process also concerned which was insensitive to siliconed glass and perhaps also to perspex. This proved to be an important point and further reference is made to it below.

The visible features of the spreading surface-change of the thrombocytes, and of another leucocyte which may play a part in the coagulation of toad blood, will be described in detail in the next paper in this series (Hackett and LePage, 1961c).

Tait (1918) showed that when a film of frog's blood clotted, a net of fibrinous material was formed, with agglutinated thrombocytes at the nodes of the net. Kopsch (1901) and Tait and Burke (1926) observed a similar phenomenon with mammalian platelets. We attempted the same experiment with the toad but found that its "fibrin" was so jelly-like and non-fibrous that it was not possible to see by phase-contrast microscopy whether or not its thrombocytes acted as visible centres of clot precipitation.

Glass activation of a non-cellular plasma factor.

Using only test tubes and pipettes coated with paraffin wax, we then prepared saline leucocyte suspensions, supernatant saline extracts of leucocytes,

and citrated cell-free plasma. We allowed a sample of each to have a short (120 secs.) period in a plain glass test tube before being returned to wax and we then compared the effects of this temporary glass-contact on these preparations, using recalcified clotting times in waxed tubes as indicators. Glass-contact enhanced the thromboplastic activity of plasma-free leucocytes and it also initiated a co-thromboplastic activity in leucocyte-free plasma.

We then extended the experiment to compare the contact activation properties of plain glass with siliconed glass. We found that siliconed glass did not promote the plasma co-thromboplastic effect, but its influence on leucocytes was equal to that of plain glass. The "silicone anomaly" referred to above could therefore be explained by assuming that there was in the toad's blood a glass-activated non-cellular plasma co-factor of leucocyte thromboplastin, and that this co-factor was not activated by siliconed glass. The toad leucocyte thromboplastin, on the other hand, was activated not only by plain glass but also by siliconed glass.

We then found that mixtures of glass-activated plasma with plain- or siliconed-glass-activated leucocytes would achieve optimum intrinsic thromboplastic activity only if the mixture itself was then put in contact with plain (but not with siliconed) glass for a short period.

Further experiments showed that perspex was equivalent to siliconed glass in all the effects described above.

These surface activations of the plasma factor and of the leucocytes, and of their subsequent interaction, were all found to be independent of the presence of calcium ions as they could take place in the presence of decalcifying anticoagulants.

Serum accelerator and serum anticoagulant.

Serum is perhaps an unphysiological clotting reagent, being extracted only after coagulation has taken place, and its effects *in vitro* on unclotted plasma or on leucocytes may then be artefacts. However, serum is possibly the simplest, or only, source of stable enzyme-like factors which are not consumed quantitatively in the clotting reaction, particularly if they are finally formed only during the course of the reaction. In human blood, plasma thromboplastin antecedent, Hageman factor, Christmas factor, Prower-Stuart factor and factor VII can all be distinguished in serum.

In toad serum we found an accelerating factor which reduced the recalcified clotting time of either leucocyte-rich or leucocyte-free plasma but which did not alter the recalcified clotting time of the same plasma with added tissue-extracts. Two-stage tests showed that the addition of the toad serum accelerator enhanced the rate of maximum thrombin production in leucocyte-rich plasma but did not do so in plasma with added fresh toad brain thromboplastin diluted to give a thromboplastic activity equivalent to the leucocytes. Accelerating serum did not restore or enhance the recalcified clotting time of alumina-

adsorbed toad plasma with or without leucocytes. The serum accelerator was itself adsorbable with alumina and was inactivated in 15 mins. at 56° C. We concluded from these experiments that the accelerating effect was due neither to the presence of prothrombin or thrombin in the serum, nor to the carry-over of leucocyte thromboplastin which is relatively heat stable (Hackett and LePage, 1961c).

The serum accelerator required calcium ions for its effect on plasma-free leucocytes but not for its effect on leucocyte-free plasma. Serum which had been formed in the presence of excess glass surface (glass powder) had no more accelerator activity than serum formed in a plain glass test tube.

In fresh serum, separated immediately after clot retraction had begun, the serum accelerator was obscured by an anticoagulant. This anticoagulant was labile and disappeared in a few hours, while the accelerator was relatively stable and persisted so that its effects could be studied without difficulty in stored serum. Experiments by pre-incubation in turn with toad leucocytes, toad plasma, and bovine thrombin, showed that the anticoagulant acted independently of the presence of calcium ions and was not an anti-fibrinogen or anti-thromboplastin but appeared to be mainly an anti-thrombin in its effects on a recalcified plasma clotting time. It differed from the fibrinolysin described in the previous paper (Hackett and LePage, 1961a).

DISCUSSION.

"Completeness" of thromboplastins.

Intrinsic cell-thromboplastic factors in human blood are provided by platelets. These are non-nucleated fragments of cytoplasm derived from megakaryocytes. They, or their products, react with plasma and serum co-factors to form complete intrinsic blood thromboplastin. If a simple extract of tissue (e.g. brain) is added to human plasma, clotting will take place without the action of several of the plasma and serum co-factors of platelet activity. A tissue extract, being derived from whole cells, might be expected to contribute a greater range of thromboplastic reagents than that provided by reduced cell fragments such as platelets. The fact that the thromboplastic action of tissue extract ("extrinsic thromboplastin") on human plasma is independent of some of the non-cellular plasma components of human intrinsic thromboplastin can be taken as an indication that such an extract is indeed thromboplastically more "complete" than platelets.

The general theories of Heilbrunn (1934, 1937, 1956) have had attention drawn to them in connection with blood coagulation by Robb-Smith (1955). Heilbrunn suggests that plasma clotting in the higher animals is the result of a progressive evolutionary extension into the extracellular plasma of the cytoplasmic sol-gel mechanism which is present in the cells of all living organisms. This mechanism underlies such activities as protoplasmic streaming, cell divi-

sion, amoeboid movement, phagocytosis and muscle contraction. It must have been developed and stabilised as a cell mechanism long before the evolution of complex multicellular organisms.

Bufo marinus is a poikilothermous animal with a low arterial blood pressure. As an amphibian it represents one of the earlier stages of evolving vertebrate complexity. It possesses extracellular plasma fibrinogen and prothrombin, like the higher vertebrates, but we have observed that the intrinsic thromboplastin of its blood is contributed mainly by whole cells, and not by platelets. This toad blood cell thromboplastin ought then to be more "complete", comparatively speaking, than that which derives from human blood platelets. We report above that our tests confirm that this is so. Toad "intrinsic" thromboplastic activity can be compared more closely with that of human "extrinsic" or tissue whole-cell thromboplastin than with platelet thromboplastin. Accordingly, Heilbrunn's hypothetical evolutionary extension of the cytoplasmic clotting mechanism into the extracellular plasma seems not to have proceeded as far in this more primitive vertebrate as it has in the mammals, which is what one would expect.

At the same time, toad leucocyte thromboplastin is not, even when the cells are concentrated, as fully effective as the most active toad tissue preparations (Tables 1 and 2). Therefore it may be that, though very "complete" compared to human platelets, toad leucocyte thromboplastin is not entirely independent of extracellular thromboplastic co-factors in plasma or serum. Our results quoted in this paper show that there is one such factor in toad plasma and one in toad serum.

Comparison between toad and human plasma and serum factors.

A useful one-for-one empirical comparison between toad and human non-cellular blood coagulation factors can be made.

Prothrombin seems to have very similar properties in both species. The difference in quality between toad and human fibrinogens can be regarded as a minor one from a broad biological point of view, in that both produce a plasma gel.

The toad plasma co-thromboplastic factor is activated by glass and is not dependent on the presence of calcium ions. Therefore, when compared with the two known human contact factors, it is (being independent of calcium) similar to Hageman factor and unlike Rosenthal's plasma thromboplastin antecedent (Soulier, Wartelle and Ménaché, 1959). We have shown in the previous paper in this series that the plasma activity of toad blood includes no labile factors comparable to human factor V or anti-haemophilic globulin.

The toad serum thromboplastic accelerator is active in the presence of intrinsic thromboplastin but does not affect extrinsic thromboplastin, and it is adsorbable with alumina. In these properties it is, compared with the human

serum factors, unlike factor VII, Prower-Stuart factor or Hageman factor and it is similar only to Christmas factor.

If this analogy with human factors is acceptable it suggests that the toad's extracellular clotting factors comprise only fibrinogen, prothrombin, a Hageman-like plasma factor, a Christmas-like serum factor and calcium ions.

In the toad, then, one plasma factor (apart from prothrombin and fibrinogen) and one serum factor can be contrasted with the four plasma factors and three serum factors accepted for human blood. It is true, of course, that human factors once thought to be single have later been shown to be multiple, and that most of the coagulation factors in human plasma and serum have been recognised only after many years of investigation and through their absence in patients suffering from rare haemorrhagic syndromes. We have examined no toads in acquired or congenital haemorrhagic states and, apart from conditions which might be induced by drugs, we doubt whether such material could be obtained.

However, the technique of measurement of intrinsic thromboplastin generation (Biggs and Douglas, 1953) has greatly clarified the field of human blood clotting studies. It is safe to say that if this experimental method had been evolved earlier the recognition of many of the individual human plasma and serum clotting factors would not have been so long delayed. We have used the same experimental approach in our investigation of toad plasma, where the relative "completeness" of toad leucocyte thromboplastin has, in fact, greatly simplified the techniques required.

Therefore we suggest that the paucity of individual non-cellular clotting components in toad plasma and serum which is suggested by the present work is real and not merely an apparent deficiency which further more enlightened or more intensive investigation would remedy.

Finally, it should be considered whether the toad's leucocytes may perhaps contain some intracellular agents comparable to the human extracellular clotting factors of which the counterparts in toad plasma and serum are absent. We have no evidence yet that this is so, but the view is in general accord with Heilbrunn's concept of the evolution of extracellular clotting mechanisms. Consumable factors similar to human anti-haemophilic globulin or factor V might be found inside toad cells. Enzyme-like non-consumable factors such as plasma thromboplastin antecedent or Prower-Stuart factor which act on other previously inert components might not be present inside the cell at all if the cell-wall is a sufficient barrier and trigger to control the action upon plasma of the intracellular consumable factors. On the other hand, the visible changes of "viscous metamorphosis" in toad thrombocytes may reflect the same chains of enzyme-substrate reactions occurring intracellularly as take place extracellularly in mammalian plasma in the early stages of blood coagulation.

A scheme for the clotting mechanism of toad blood, derived from the conclusions of this and the previous paper, is given in Fig. 1.

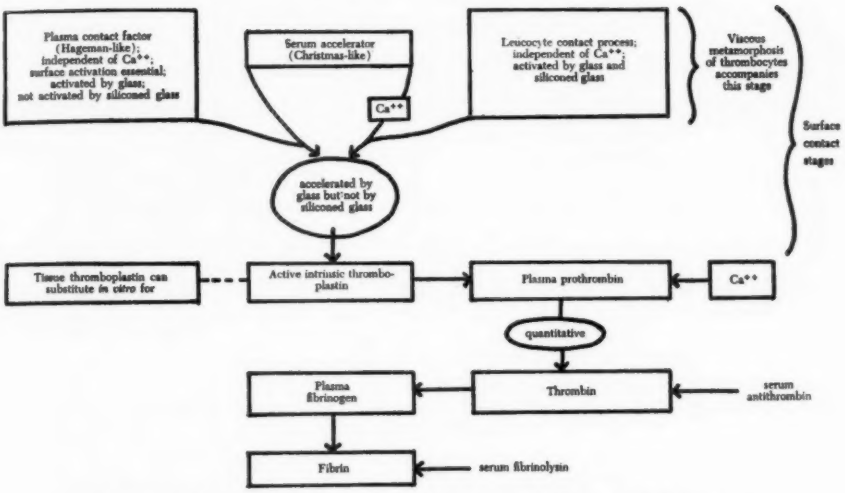

Plasma contact factor (Hageman-like); independent of Ca++; surface activation essential; activated by glass; not activated by siliconed glass
Serum accelerator (Christmas-like)
Ca++
Leucocyte contact process; independent of Ca++; activated by glass and siliconed glass
Viscous metamorphosis of thrombocytes accompanies this stage
Surface contact stages
accelerated by glass but not by siliconed glass
Tissue thromboplastin can substitute in vitro for
Active intrinsic thromboplastin
Plasma prothrombin
Ca++
quantitative
Plasma fibrinogen
Thrombin
serum antithrombin
Fibrin
serum fibrinolysin

Fig. 1. Scheme for the blood coagulation mechanism of *Bufo marinus*.

Acknowledgments. We are grateful for the expert technical help and advice of Mr. John Marshall. We wish to thank Dr. J. A. Bonnin and Dr. N. D. Hicks for many discussions in which they placed their long experience of human blood coagulation problems at our disposal. Miss Maureen Dangerfield gave valuable technical assistance.

REFERENCES.

Biggs, R., and Douglas, A. S. (1953): J. clin. Path., 6, p. 23.
Biggs, R., and Macfarlane, R. G. (1957): *Human blood Coagulation and its Disorders.* Blackwell, Oxford.
Hackett, E., and LePage, R. (1961a): Austral. J. exp. Biol., 39, p. 57.
Hackett, E., and LePage, R. (1961c): In preparation.
Heilbrunn, L. V. (1934): Biol. Bull., 66, p. 264.
Heilbrunn, L. V. (1937): *An Outline of General Physiology.* Saunders, London.
Heilbrunn, L. V. (1956): *The Dynamics of Living Protoplasm.* Academic Press, N.Y.
Kopsch, F. (1901): Anatom. Anz., 19, p. 541.
Pringle, H., and Tait, J. (1910): *J. Physiol.*, 40, p. 35.
Pringle, H., and Tait, J. (1911): *Ibid.*, 42, p. 38.
Robb-Smith, A. H. T. (1955): Brit. med. Bull., 11, p. 70.
Sharp, A. A. (1958): Brit. J. Haemat., 4, p. 28.
Soulier, J. P., Wartelle, O., Ménaché, D. (1959): *Ibid.*, 5, p. 121.
Tait, J. (1918): Quart. J. exp. Physiol., 12, p. 1.
Tait, J., and Burke, H. E. (1926): Quart. J. exp. Physiol., 16, p. 129.

SOME QUANTITATIVE STUDIES ON TISSUES OF LAMBS DWARFED BY HIGH TEMPERATURES DURING GESTATION

by MARGARET RYLE[1] AND L. R. MORRIS[2]

(From the Departments of Animal Husbandry and Physiology, University of Queensland).

(*Accepted for publication 20th December, 1960.*)

SUMMARY.

Quantitative studies were made on the anterior pituitary, thyroid, adrenal and bladder epithelium of normal new-born lambs and lambs from ewes exposed to high environmental temperatures during the last two-thirds of pregnancy.

The mean relative weights of the anterior pituitaries and of the adrenals of the dwarfed hot-room lambs were significantly greater than those of the controls.

Where the experimental treatment caused dwarfing, the pituitary cell size was less than in the controls and intensely chromophilic nuclei were more frequent. However, the total number of anterior pituitary cells did not appear to be affected. The area and height of the nuclei (and cells) and the number of cell layers in the bladder epithelium were also reduced in the dwarf lambs.

Regardless of body weight, the hot-room lambs tended to have smaller adrenal cortices, and the mean area of the nuclei in the zona fasciculata was less than that of the controls. The quantity of adrenal medulla, however, appeared to be unaffected by the treatment, even where dwarfing occurred.

No significant differences were detected between the thyroids of the hot-room animals and those of the controls.

The results are discussed in relation to possible mechanisms through which high environmental temperatures might affect the tissues studied.

INTRODUCTION.

Yeates (1958) showed that dwarf lambs obtained from ewes exposed to high environmental temperatures during pregnancy were distinct from the light-weight, but skeletally normal young which result from maternal malnutrition. Macfarlane *et al.* (1959) suggested that the dwarfing might be due to "placental defect, reduced blood supply, pituitary insufficiency, or adrenal steroid excess" resulting from excessive heat. Little information appears to be available concerning the effects of this kind of stress on the cellular morphology of

---

[1] George Aitken Pastoral Research Trust Research Fellow. Present address: Department of Physiology, University of Melbourne, Parkville, N.2.

[2] Supported by Wool Research Trust Fund. Present address: Department of Zoology, University of Wisconsin, Madison, Wis., U.S.A.

the anterior pituitary, of the adrenal or of any other tissues in any species. Recently, as a by-product of an investigation designed primarily to study the effects of high temperatures on lactation (Morris, in preparation), the opportunity arose for some histological work on dwarf lambs and on comparable controls.

Material and Methods.

A small number of Romney ewes was obtained from each of three flocks. Sheep from each flock were distributed at random between the hot-room and control groups. At first all ewes were kept in a yard pen with one open side. Each was mated with the same Romney ram. One-third of the way through pregnancy four ewes were put in the hot-room. At parturition, they and their lambs were returned to the animal yard. The nine control ewes were kept in a yard pen throughout the experiment. The four hot-room ewes produced five lambs (one pair of twins) of which only one was a female. Four were dwarfs of the type described by Yeates (1958). The nine control ewes produced twelve lambs (three pairs of twins) of which six were females and none were dwarfs.

In the hot-room the heaters were switched on for 8-9 hours per day and the room reached its maximum temperature of 40·6° C. (105° F.) in approximately 2-3 hours. The wet-bulb temperature was set at 32·2° C. (90° F.), i.e. 56 p.c. relative humidity. The time switch regulating the lights in the hot-room was adjusted weekly to give the same average day-length as that received by the controls. Each group of ewes was given a daily allowance of 2¾ lb. per head of good quality lucerne chaff, all of which was regularly consumed in both pens. Water was always available. Weight gains during pregnancy and the duration of pregnancy are shown in Table 1.

Gestation periods tended to be slightly shorter in the hot-room but none of the lambs was truly premature although the one born at 141 days was still-born. All the rest were weighed and killed with ether within 24 hours of birth (usually within 12 hours). Except in the case of the still-born lamb, where dissection was inevitably delayed, the anterior pituitary,

TABLE 1.

Weight gain of ewes and duration of pregnancy.

	Ewe No.	Weight gain (kg.)	Duration of pregnancy (days)
Controls	203 (T)	13·1	147
	215	13·8	148
	219 (T)	13·9	145
	230 (T)	9·1	147
	237	8·2	147
	241	9·4	150
	247	9·5	146
	255	12·1	149
	261	9·4	149
	Control Mean:	10·9	147·6
Hot-room	217	6·3	141
	223 (T)	10·3	147
	228	12·0	147
	257	10·5	144
	Hot-room mean:	9·8	145·0

(T) = twins.

adrenal and thyroid glands were removed immediately after death and fixed in 10 p.c. formal saline neutralized with calcium carbonate. The adrenals and thyroids were slit after trimming away adhering fat, mesentery etc., to permit better penetration of the fixative. In order to examine some non-secretory epithelial tissue, transverse slices taken halfway along the bladder were fixed similarly. Since the tissues usually made the fixative acid it was changed once or twice within the first hour and again after about 24 hours. All tissues were in formal saline for at least two weeks. Before dehydration in graded alcohols the glands were blotted gently with filter paper and weighed, the combined weights being recorded for the two thyroid lobes and for the two adrenals. The pituitaries and bladder samples were cleared with xylol, but the thyroids were cleared with benzene and the adrenals with cedar-wood oil since xylol hardened these tissues too much. Embedding was carried out in 56° M.P. paraffin wax.

All tissues were cut at 5μ. The anterior pituitaries were sectioned sagitally, one thyroid lobe and one adrenal from each lamb was sectioned transversely about halfway along the glands and the bladder sections were approximately transverse. Sections of all tissues were stained with Harris's haematoxylin and counterstained with Orange G. Throughout, slides bearing sections of a given tissue from every lamb were placed in a rack and treated simultaneously, thus reducing the likelihood of differences arising from variations in the processing.

The tissues were examined in the following manner:

Pituitaries.

In order to compare cell size under the two experimental conditions the number of nuclei in a given area was counted with the aid of an eyepiece graticule, this figure being an inverse function of cell size. Two non-adjacent sections of each pituitary were processed on separate slides at different times. The nuclei in six widely separated areas of each section were counted. Areas lacking large blood vessels were selected in the peripheral zone of the sections, where most acidophils occurred, since the available evidence indicates that acidophils produce growth hormone. The sum of the nuclei in the twelve areas was recorded for each lamb.

In order to compare nuclear size, ten nuclei were measured in each of four areas within a single section. To avoid subjective selection ten consecutive nuclei lying on a randomly chosen straight line were measured. Again the areas selected were within the more peripheral zone and were without large blood vessels, but they were not identical with those used for nuclear counts. The nuclei were measured in arbitrary units by means of an eyepiece micrometer (1 unit = 1·13μ). The maximum diameter was measured and also that at right angles to it approximately halfway along its length. Although some nuclei were not regular ellipses, the product $\frac{\text{Diameter}_1}{2} \times \frac{\text{Diameter}_2}{2}$ provides an approximate index of the area of nuclear cross-section. The mean area index for each lamb's pituitary was calculated from its 40 measured nuclei. The proportion of strongly chromophilic nuclei among those measured was also noted.

Adrenals.

Nuclear counts were made by the same method in the zona fasciculata of the cortex. The number of fields examined was increased to ten per section because mitoses were common in some of the adrenals and a more reliable estimate of their frequency was desired.

Nuclei of the zona fasciculata were measured as in the case of the pituitaries, care being taken to avoid areas in which large blood vessels, much connective tissue or numerous necrotic nuclei occurred. If necrotic nuclei or those undergoing mitosis occurred along the

line selected, these were omitted. The mean nuclear area index was calculated for the adrenal of each lamb.

The maximum and minimum diameters of a complete section of the adrenal gland, and of the medulla in the same section, were determined in arbitrary units. Indices for the cross-sectional areas of the whole gland and of the medulla were calculated in the same way as those for the cell nuclei. The cortical area index was obtained as the difference.

Thyroids.

Unfortunately the staining method did not permit accurate measurement of the height of the thyroid epithelial cells. Nuclei, however, were measured and their height, perpendicular to the plane of the epithelium, appeared to vary with cell height. Within a single section 10 of the larger follicles were selected; these were widely separated from one another. Occasional exceptionally large follicles were excluded. Smaller follicles were avoided to reduce the risk of tangential sections. Within each of the selected follicles the height and width of the nuclei of five adjacent cells were determined. The number of nuclei measured in this tissue was increased to 50 because the histology of the thyroid is notoriously variable (c.f. Myers and Ross, 1959). Normally the longest axis of the nucleus was approximately either perpendicular to or parallel with the plane of the epithelium, but when not so orientated the longest axis was considered to be vertical if less than 45°, and horizontal if more than 45° from the perpendicular position. The ratio of height to width provides an index of nuclear shape and an indication of cellular shape. Both mean area and mean shape indices were calculated for the nuclei of each lamb's thyroid.

The number of follicles per unit area of the thyroids and the frequency of vacuoles in the colloid were also investigated.

Bladder epithelium.

Nuclei of the basal layer of the bladder epithelium were measured in the same way as those of the thyroid epithelium. Ten adjacent nuclei were used in each of four widely separated regions within a single section. Straight regions were selected since, in transitional epithelium, folds greatly distorted the form of the cells and their contents by compression or extension. Mean nuclear shape and mean nuclear area indices were calculated for the bladder epithelium of each lamb.

The average number of cell layers in the bladder epithelium was estimated by counting the number of overlying nuclei at six widely separated points. These were in straight stretches of a single section of the bladder wall.

In examining the results no differences due to the flock from which the ewe was obtained, the sex of the lamb, or single versus twin status were obvious. These possible sources of variation have therefore been ignored. Data relating to the one hot-room lamb which was not dwarfed (that of Ewe No. 257) are shown separately in all the following Tables. The significance of the difference between the mean for the four hot-room dwarfs and that for the twelve control lambs has been determined in every case by the usual 2-sample t-test.

RESULTS.

Body weight and relative organ weight.

Table 2 contains data on the weight of the lambs and the relative weights of their anterior pituitaries, adrenals and thyroids. Five of the anterior pituitaries were damaged during dissection; only the weights of the undamaged ones from 8 controls and 3 hot-room lambs have been used in calculating the mean pituitary

weight/body weight ratios. The dwarfing effect of the hot-room treatment is indicated by the marked difference between the mean body weights. The exceptional hot-room lamb, No. 257, was well within the control range. Its dam underwent similar treatment the previous year, and in this instance also the weight of her lamb was within the control range, as was her milk production (Morris, in preparation). It is possible, of course, that under normal conditions this ewe would produce an exceptionally heavy lamb and high milk yield.

TABLE 2.

Mean body weight and mean relative weight of anterior pituitary, adrenals and thyroid.

	Hot-room		Controls	
	No. 257	Dwarfs Mean S.E.	Mean S.E.	P
Body weight (kg.)	3·6	2·02±0·30	4·07±0·24	<0·001
Ant. pit. wt. (mg.) / Body weight (kg.)	28·6	32·0±1·1	23·1±1·3	<0·01
Adrenal wt. (mg.) / Body weight (kg.)	161	260±33	174±5	<0·001
Thyroid wt. (mg.) / Body weight (kg.)	186	265±38	287±23	>0·05

In view of the marked difference in mean body weight it is not surprising that the pituitaries, adrenals and thyroids were smaller in the dwarf lambs than in the controls. However, if *relative* weights are considered, the mean for the hot-room dwarfs' anterior pituitaries is highly significantly greater than that for the controls; so also is the mean relative weight of the adrenals. On the other hand, the mean relative weights of the thyroids do not differ much, although in adult animals depressed thyroid activity is a characteristic response to a hot environment.

Anterior pituitaries.

Preliminary inspection suggested that the nuclei of the hot-room lambs' anterior pituitaries lay closer together than those of the controls. Therefore, systematic counts were undertaken as described previously. Table 3 shows that the mean number of nuclei per unit area in the dwarfs was highly significantly greater than that in the controls. However, hot-room lamb No. 257, which was of the normal size, had a count lower even than the control mean. As well as cell size, the nuclear area appeared to be reduced by the heat treatment but the difference between the mean nuclear area index of the dwarf hot-room lambs and that of the controls was not significant. On the other hand the average proportion of intensely staining nuclei was doubled. Lamb 257 was

TABLE 3.

Anterior pituitary cells and nuclei.

	Hot-room		Controls	
	No. 257	Dwarfs Mean S.E.	Mean S.E.	P
No. nuclei/unit area	880	1246±79	959±28	<0·001
Nuclear area index	7·14	4·63±0·20	5·60±0·26	>0·05
% intensely staining nuclei	7·5	22·5±1·0	10·6±2·2	<0·01

again exceptional. Thus the changes observed in the pituitary cells and nuclei were characteristic of the dwarfed lambs: where heat exposure did not induce dwarfing, it did not result in these changes.

Since the anterior pituitary in the hot-room lambs was relatively heavier than normal, yet the cells were smaller, it seemed of interest to calculate comparative indices of the number of cells in these glands. If the number of cells per unit area of an infinitely thin section equals N, $N^{3/2}$ would give the number of cells per unit volume, provided they were uniform and symmetrical. $N^{3/2} \times$ total volume of the gland would then give the total number of cells in the

TABLE 4.

Estimated comparative index of the number of anterior pituitary cells.

	Body wt. (kg.)	Ant. pit. wt. (mg.)	Pit. wt. (mg.) / Body wt. (kg.)	Nuclei/unit area	Comparative index of total no. of cells
Controls	3·6	72	20·0	1081	256
	4·9	107	21·8	852	266
	3·7	81	21·9	904	220
	3·2	75	23·4	902	203
	4·5	96	21·3	824	227
	4·6	102	22·2	1031	338
	4·7	104	22·1	983	321
	4·7	149	31·7	804	340
Con. mean	4·24	98·3	23·0	923	271
Hot-room dwarfs	1·6	53	34·2	1099	193
	1·7	53	31·2	1466	298
	1·9	58	30·5	1170	232
Dwarf mean	1·73	54·7	32·0	1245	241
Dwarf mean as % of control mean	40·8	55·6	139·1	134·9	88·9
No. 257	3·6	103	28·6	880	269

N.B. Lambs with pituitaries damaged during dissection are excluded from this Table.

latter. The nuclear counts provide estimates of the number of cells per unit area. In place of volume we have the weight of the gland, which, provided no differences of density occur, should be a function of volume. Thus an approximate comparative index of the number of cells per gland can be calculated. Table 4 shows these indices, together with other data for the twelve lambs of which the pituitaries were undamaged. Lambs for which satisfactory pituitary weights are not available are excluded. The hot-room dwarf mean is expressed as a percentage of the control mean for each index. Whereas the hot-room mean body weight is less than half that of the controls, the estimated number of pituitary cells is only 11 p.c. less. Moreover, although hot-room lamb No. 257 is exceptional with respect to body-weight, pituitary weight and nuclei per unit area, it appears to have about the same number of pituitary cells as the other hot-room animals.

Adrenals.

Although the mean relative weight of the adrenals, like that of the pituittaries, was greater in the dwarf hot-room lambs than in the controls, the mean number of nuclei per unit area of the zona fasciculata was not significantly greater (Table 5). That is, dwarfing was not associated with a marked diminu-

TABLE 5.
Adrenal cells and nuclei.

	Hot-room		Controls	
	No. 257	Dwarfs Mean S.E.	Mean S.E.	P
No. nuclei/unit area	1254	1028±85	980±43	>0·05
Nuclear area index	7·82	7·72±0·35	8·89±0·24	<0·05

tion in the size of the cortical cells. No. 257, it should be noted, had the smallest cortical cells of any lamb. In contrast, the mean nuclear area index was significantly less in the dwarfs than in the controls; Lamb 257 again resembled the hot-room animals rather than the controls in this respect.

A striking feature of the nuclei of the zona fasciculata in some of the glands was the high proportion of mitoses. Up to 1·3 p.c. metaphase plates were counted. This actively dividing condition was, however, correlated neither with the experimental treatment nor with the time between birth and death. Even within a pair of twins, one might have numerous mitoses and the other few or none.

Although the mean relative weight of the gland was greater in the dwarf hot-room lambs, the mean absolute weight was less; 498 ± 39 mg. compared with 707 ± 46 mg. ($P < 0·05$). It has tentatively been assumed that the in-

crease in relative weight is associated with increased cortical activity (Yeates, 1958). However, reduced nuclear size in the adrenal cortex is indicative of reduced activity (Miller, 1954). It was therefore of interest to determine how the proportion of the total gland occupied by the cortex was altered by the hot-room treatment. As Table 6 shows, the cortex constituted significantly less

TABLE 6.

Adrenal cortex and medulla.

	Hot-room		Controls	
		Dwarfs		
	No. 257	Mean S.E.	Mean S.E.	P
Cortex as % of cross section of gland	61·6	55·5±2·4	65·7±1·7	<0·01
Cortex: area index	12·5	11·3±0·9	18·4±1·9	<0·05
Medulla: area index	7·79	9·05±0·90	9·25±0·71	>0·05
Estimate of weight of medulla	22·25	21·95±1·34	23·75±1·47	>0·05

of the cross-sectional area of the gland in the hot-room dwarf lambs than in the controls. The cortical area indices themselves also differ significantly. Since cell size was not significantly reduced, this suggests a diminution in the number of cortical cells in the hot-room. The medulla area indices, on the other hand, are not significantly different. Nevertheless, since the glands themselves vary in shape, some being relatively long and thin and others short and fat, this does not necessarily imply that the total quantity of medullary tissue is on the average the same for hot-room and control lambs.

Measurements were made of the adrenals, not used in preparing the sections, in order to detect possible consistent differences of shape between the hot-room and control groups. Length and the maximum and minimum breadth halfway along the gland were determined after the material had been approximately five months in formal saline. During this time all the glands had decreased somewhat in weight, but it is assumed that any linear shrinkage was proportionately the same for all. The cross-sectional area index was obtained in the usual way and the ratio between the length and this index was calculated for each lamb. The average ratio for the control lambs was $1 \cdot 38 \pm 0 \cdot 06$; that for the dwarf hot-room lambs was $1 \cdot 39 \pm 0 \cdot 20$. The difference is clearly not significant and there is no reason for supposing that the shape of the glands was affected by the experimental treatment. This suggests that the quantity of medulla was not in fact affected by the heat exposure. Unfortunately, an accurate comparison of the volume of medullary tissue cannot be made. Nevertheless, if it is assumed that the gland consists of a regular cylindrical rod of medulla surrounded by a hollow cylinder of cortex, and that the densities of the two tissues are the same, an approximate estimate of the weight of medullary tissue can be obtained: i.e.

the medullary area, expressed as a fraction of the entire cross-section, multiplied by the weight of the gland. The difference between the mean estimated weights for the two groups of lambs does not reach the 5 p.c. level of significance. It therefore appears that the difference in absolute weight of the adrenals is due primarily to the reduction of cortical tissues in the hot-room animals. The greater relative weight results from the apparently *normal* development of the medulla which, in view of the general dwarfing of most parts of these animals, is perhaps the most interesting feature of all. With respect to the amount of adrenal cortex, No. 257 again resembled the other hot-room lambs more than the controls, suggesting that adrenal cortical function is more consistently affected by heat exposure than is the pituitary cell size or growth rate of the foetus.

Thyroids.

Just as there was no consistent effect on the relative weights of these glands, the experimental procedure failed to demonstrate any consistent difference in the size of the thyroid follicles or the frequency with which vacuoles appeared in the colloid. Although in the epithelium the hot-room mean nuclear area index was less than that of the controls, the difference was not significant (Table 7). The nuclei of the hot-room animals were, on the average, slightly more

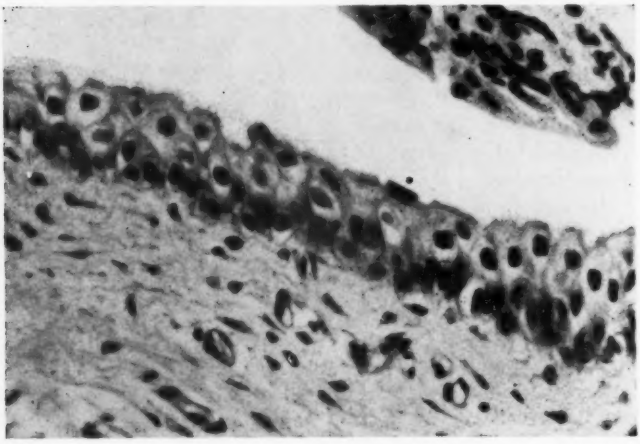
TABLE 7.
Thyroid epithelium nuclei.

	Hot-room		Controls	
		Dwarfs		
	No. 257	Mean S.E.	Mean S.E.	P
Nuclear area index	4·29	3·80±0·30	4·38±0·17	>0·05
Nuclear shape index.	0·671	0·659±0·060	0·693±0·043	>0·05
Nuclear height	3·34	3·13±0·27	3·44±0·17	>0·05

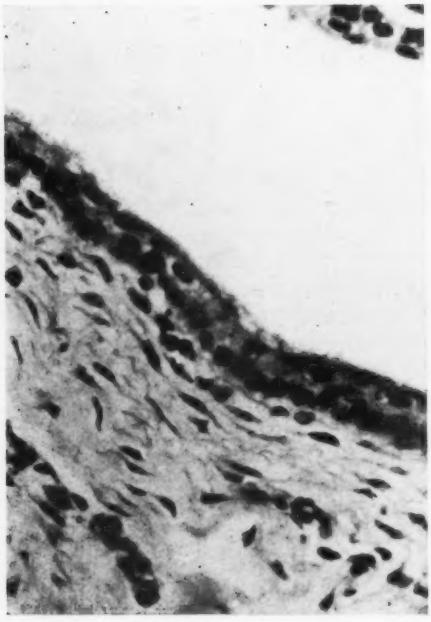
flattened, but neither the mean shape index (height/width) nor the mean height of the hot-room dwarfs' nuclei differed significantly from the control measurements. The outstanding characteristic of the thyroid tissue was its extreme variability, not only within a uniformly treated group of animals, but even within a single section of one gland.

Bladder epithelia.

The bladder epithelium, in contrast to the thyroid epithelium, was markedly affected by the hot-room exposure (Table 8). Firstly, the mean nuclear area index was significantly less than that of the controls. Secondly, the nuclei and the cells were much more flattened. Above all, however, the mean number of cell layers was highly significantly reduced (Fig. 1). As in the cases of



1



2

3

Fig. 1. Bladder epithelia. 1. Control lamb.
2. Live dwarf hot-room lamb.
3. Still-born dwarf hot-room lamb.

TABLE 8.

Bladder ephithelium nuclei and cell layers.

	Hot-room		Controls	
	No. 257	Dwarfs Mean S.E.	Mean S.E.	P
Nuclear area index	6·92	5·45±0·23	6·76±0·26	<0·02
Nuclear shape index	1·69	0·70±0·04	1·34±0·07	<0·001
No. cell layers	4·00	1·71±0·28	3·17±0·21	<0·01

body weight and pituitary cell morphology, Lamb 257 was exceptional in these features and resembled the controls rather than the hot-room dwarfs. The amounts of muscle and connective tissue, as well as the number of epithelial cell layers, were reduced in the dwarf lambs, but quantitative treatment of these tissues is more difficult and has not been attempted.

DISCUSSION.

Reduced pituitary cell size and attenuated bladder epithelium were associated with dwarfing and in these respects Lamb 257 differed from the other four hot-room animals. On the other hand, in all hot-room lambs, regardless of body size, the adrenal cortex and the nuclear size in the zona fasciculata were reduced. This distinction suggests either that different tissues may be affected by heat through different intermediary mechanisms, or that they differ in susceptibility to a single one.

It is of interest that the anterior pituitary cells (most of which are basophils or neutrophils) of genetically dwarf mice are also unusually small (Elftman and Wegelius, 1959). Solomon and Greep (1959) found that the weight of the anterior pituitary was reduced in rats following thyroidectomy; fewer acidophils were to be found and the content of growth hormone decreased. Thyroxine therapy restored the growth hormone level. However, in the present work the thyroids showed no significant response to the hot-room treatment. While recognizing that the histological picture alone is an unreliable index of thyroid activity, the results of Srebnik, Nelson and Simpson (1959) may be more relevant. They showed that in protein deficient rats the anterior pituitary decreased in weight, had fewer acidophils and less growth hormone, and in addition that the cells were smaller than normal. It has recently been demonstrated (Graham *et al.*, 1959) that at elevated temperatures the protein catabolism of sheep is enhanced. It is possible that when a ewe was put in the hot-room the supply to her embryo of one or more essential amino-acids declined, or alternatively that the requirements of the embryo itself increased to a point where the supply was inadequate. This in turn might prevent normal synthesis

of growth hormone by the embryonic pituitary and hence result in limited mitosis and reduced growth rate generally. It could perhaps account for the inadequate bladder epithelium. On the other hand, as suggested elsewhere (Ryle, 1960), raised body temperature may result in the supply of vitamin A being inadequate for the demands of the foetus. Squamous metaplasia and keratinization have been reported in a number of tissues, including the bladder of the monkey (Tiden and Miller, 1930), as a result of vitamin A deficiency. Although no keratinization is apparent, it is clear from the photographs and from the nuclear shape data that the epithelial cells of the bladder are reduced in height in the dwarfed lambs.

According to Cater and Stack-Dunne (1953) hypophysectomy results in diminished thickness of the rat's adrenal cortex, especially of the zona fasciculata, and both ACTH and growth hormone partially restore it. Miller (1954) showed that hypophysectomy resulted in a marked reduction of nuclear and nucleolar size in the zona fasciculata. Injections of ACTH increased their size in operated rats, though they did not reach the control dimensions. Thus the changes observed in the lambs' adrenals tend to confirm the suggestion (Macfarlane *et al.*, 1959) that heat exposure leads to general pituitary inadequacy, which, if its cell numbers are constant, is a functional deficiency, not a developmental one. Nevertheless, the apparent dissociation of adrenal response from overall growth response in Lamb 257 suggests that the depression of ACTH production does not have the same immediate cause as that of growth hormone production. Holland (1958) found that foetal rat adrenals weighed less when the pregnant females were exposed to stress conditions (low atmospheric pressure). He suggested that increased concentrations of maternal cortical hormone circulating on both sides of the placenta inhibited foetal ACTH production and hence normal development of the foetal cortex. This could be true of the lambs under discussion during the early part of the ewes' exposure to high temperatures, but only before they were acclimatized; continued exposure appears to depress steroid output of the ewe's cortex (Robinson and Morris, 1960). Consequently a more direct effect of heat on the lamb's ACTH production seems probable and the response may simply represent an adaptation to environmental conditions comparable to that of its dam.

It has been shown here that, despite the smaller size of the pituitary, the total number of its cells was not diminished by the hot-room treatment. It has also been shown that, even where general dwarfing occurred, the adrenal medulla developed to approximately its normal size. One might conclude that both these tissues differentiate sufficiently early, and are later morphologically sufficiently independent of other organs, to be unaffected by a hot-room exposure not beginning until one-third of the way through pregnancy. However, Yeates (1958) obtained unexpectedly heavy adrenals in dwarf lambs although the ewes were put in the hot-room immediately after mating, which suggests that even under these conditions the medulla manages to approach its normal size.

No evidence is available to indicate whether or not pituitary cell number would also be normal.

The reason for the lack of marked differences between the thyroid tissue of the hot-room lambs and that of the controls remains uncertain. It cannot be ascribed to foetal dependence on hormone supplied by the ewe since in sheep, according to Annison and Lewis (1959), the foetal thyroid is functional at least during the last third of pregnancy. The lack of any effect is the more remarkable in view of the depressed thyroid activity typical of the adult when exposed to high temperatures.

As mentioned in the introduction to this paper Macfarlane *et al.* (1959) suggested four possible means by which a hot environment might result in dwarf lambs. Regarding these, no data in the present work can indicate whether a placental defect or a reduced blood supply is implicated. However, the results, limited as they are, do indicate definite changes in anterior pituitary cell morphology; these would be consistent with depressed activity, including decreased growth hormone production. The adrenal cortical morphology also indicates that its activity is reduced in the foetus, as in the ewe, and does not support the view that excessive steroid production is involved in the diminished growth.

Acknowledgments. We wish to thank Dr. R. Tucker for advice on histological questions, Mr. E. W. Hollywood, who is responsible for the micrographs, and Professor O. E. Budtz-Olsen for his comments and criticism of the manuscript.

REFERENCES.

Annison, E. F., and Lewis, D. (1959): J. agric. Sci., 52, p. 79.
Cater, D. B., and Stack-Dunne, M. P. (1953): J. Path. Bact., 46, p. 119.
Elftman, H., and Wegelius, O. (1959): Anat. Rec., 135, p. 43.
Graham, N. McC., Wainman, F. W., Blaxter, K. L., and Armstrong, D. G. (1959): J. agric. Sci., 52, p. 13.
Holland, R. C. (1958): Anat. Rec., 130, p. 177.
Macfarlane, W. V., Pennycuik, P. R., Yeates, N. T. M., and Thrift, E. (1959): Recent Progress in Endocrinol. of Reproduction, Proc. of Conf., Syracuse, U.S.A., 1958, p. 81.
Miller, R. A. (1954): Amer. J. Anat., 95: p. 497.
Myers, B. J., and Ross, D. A. (1959): N.Z. J. agric. Res., 2, p. 552.
Robinson, K. W., and Morris, L. R. (1960): Aust. J. agric. Res., 11, p. 236.
Ryle, M. (1960): Aust. J. Sci., 23, p. 36.
Solomon, J., and Greep, R. O. (1959): Endocrinol., 65, p. 158.
Srebnik, H. H., Nelson, M. M., and Simpson, M. E. (1959): Proc. Soc. exp. Biol. Med., 101, p. 97.
Tiden, E. B., and Miller, E. G. (1930): J. Nutrit., 3, p. 121.
Yeates, N. T. M. (1958): J. agric. Sci., 51, p. 84.

PIGMENTS PRODUCED BY *MYCOBACTERIA* ON EXPOSURE TO ISONIAZID

by JEAN YOUATT

(From the School of Bacteriology, University of Melbourne.)

(*Accepted for publication 22nd December, 1960.*)

SUMMARY.

Mycobacterium tuberculosis (B.C.G.) produces pigments when exposed to isoniazid under aerobic conditions. The experimental conditions for production and separation of some of these compounds are described. Ultra-violet absorption spectra are shown. One compound was examined in more detail and some of its chemical characteristics are described.

INTRODUCTION.

When washed cell suspensions of *Mycobacterium tuberculosis* (B.C.G.) were shaken in air with isoniazid, pigments appeared in the cells and in the supernatants. An isoniazid resistant strain produced no pigment when exposed to isoniazid nor did the sensitive strain produce pigments when exposed to other anti-tuberculous drugs or the isomers of isoniazid, nicotinic and picolinic acid hydrazides (Youatt, 1958a). The production of these pigments therefore appears to be very closely related to the action of isoniazid. The present paper is mainly concerned with the methods for producing and purifying the pigments.

METHODS.

Cultures. B.C.G. was grown on the surface of a modified Sauton medium for 7 days (Youatt, 1958b). Two simple domestic articles greatly facilitated the handling of 100-150 gm. dry weight of cells used in these experiments. These were a 5-inch strainer through which all the surface cultures were poured and a "ricer" which was used to compress and remove water from the cells after they had been strained off. The "ricer" consists of a metal cup perforated with holes in the sides and base with a plunger to apply pressure from the top. The organisms were readily suspended by working them to a paste with small additions of water. They were washed by centrifugation and suspended in a suitable volume of buffer for use.

Dry weight of suspensions. At first, samples were dried rapidly under an infra-red lamp and weighed before the suspensions were adjusted to a suitable cell concentration. Later, it was found that the wet weight : dry weight ratio of 4 : 1 for the compressed cells was sufficiently reproducible for the whole mass of cells to be weighed at the end of harvesting.

Conditions of incubation. Washed cells were suspended in 0·01M phosphate buffer pH 7 at a cell concentration of 20-25 mg./ml. dry weight with 200 µg./ml. of both ammonium chloride and isoniazid. 100 ml. lots were dispensed in 500 ml. Erlenmeyer flasks and shaken at 37° C. for 24-30 hours at 90 cycles per minute with an amplitude of 2¼ inches. The supernatant was then removed and fresh buffer, isoniazid and ammonium

chloride were added in the same concentrations as above and the suspensions incubated for a further 24 hours. After removal of the cells the supernatants appeared to be stable in the refrigerator for several days, but they were usually concentrated on the following day in a rotary evaporator under vacuum below 40° C.

Cell extracts. After 24 hours' incubation with isoniazid there was a considerable amount of pigment held in the cells. Attempts to extract this met with variable success and a further 24 hours' incubation in fresh medium was more satisfactory than disruption of the cells. Extracts were prepared by boiling the cells in distilled water or dilute acid. On a small scale such extracts separated well on paper chromatograms, but on a preparative scale the other contaminating materials from the cells overloaded the paper and gave poor separation.

Purification of the pigments. Many compounds were present in the supernatants of isoniazid-sensitive B.C.G. after their exposure to isoniazid which were not present in control suspensions without isoniazid. There were unchanged isoniazid, *iso*nicotinic acid, 4-pyridyl methanol (unpublished data) and occasionally an unidentified hydrazone of isoniazid. In addition to the pigments there were other compounds which were detected by fluorescence under ultra-violet light. Paper chromatography was found to permit the sharpest separation of these products.

Samples were applied to Whatman 3 or 3MM paper and developed by ascending chromatography over 10 inches. The solvents employed were: (A), n-butanol-n-propanol-water (2:2:1); (B), solvent (A) with 5 p.c. by volume of glacial acetic acid; (C), solvent (A) with 1 p.c. by volume of ammonia sp. gr. 0·88; (D), acetone-n-butanol-water (70:20:10) and (E), n-butanol-acetic acid-water (4:1:1).

Solvents (A), (B) and (E) gave good separation of the pigments from free isoniazid, solvents (B) and (E) gave good separation from *iso*nicotinic acid and the acid solvents removed any isoniazid which was bound as a hydrazone. Neutral and acid solvents gave poor separation of the pigments and, although the compounds were less stable in alkali, no substitute for solvent (C) was found for separation on a preparative scale. A suitable sequence for the isolation of the pigments was an acid solvent, (B) or (E); alkaline solvent, (C); acid solvent; neutral solvent (A).

After the work described in this paper was completed, carboxymethylcellulose sheets became available. One of the purified pigments (compound 1, below) was retained by this paper and eluted by 1 p.c. NaCl with an R_F of 0·2. It therefore seems probable that these ion exchange papers would prove useful in the purification of the pigments.

After elution from the final chromatogram the pigment was dried, dissolved in freshly distilled absolute alcohol, filtered, concentrated to a small volume and, if necessary, centrifuged. The sample was then dried and dissolved in distilled water and centrifuged if there was any insoluble residue. It was then transferred to a weighed bottle and dried under vacuum over phosphorous pentoxide at room temperature.

Spectra. Spectra in the ultra-violet and visible regions were measured with a Unicam spectrophotometer. The infra-red spectrum of a pigment sample in a KCl disc was kindly prepared by Mr. Huntley Higgins of C.S.I.R.O., Division of Forest Products, Melbourne.

Chemicals. 2-methyl and 2-ethyl isoniazid were kindly supplied by Hoffmann La Roche (Switzerland).

RESULTS AND DISCUSSION.

Effect of experimental conditions on pigment production. The presence of oxygen was essential for pigment production. Above 25 mg./ml. dry weight of cells it was not possible to supply sufficient oxygen to the shaken suspensions.

This was also found in the measurement of the uptake of isoniazid (Youatt, 1958b). Table 1 shows the effect of cell concentration on pigment production and isoniazid breakdown.

TABLE 1.

The effect of cell concentration on pigment production.

Cells suspended in phosphate buffer pH 7 were shaken in air at 37° C. for 18 hours with 100 μg./ml. isoniazid. Colour was measured at 440 mμ. Cell concentrations are recorded in mg./ml. dry weight. Isoniazid loss was followed by a colour reaction with picryl chloride.

Cell concentration mg./ml. dry weight	Optical density at 440 mμ.	Isoniazid loss μg./ml.
30	0·00	46
27	0·10	67
24	0·17	68
19	0·12	64
10	0·07	44

The colour intensity of the supernatants increased with increasing isoniazid concentration up to 200 μg./ml. Above this concentration the colour remained as intense or fell off. 200 μg./ml. was therefore selected as the optimum for use in the experiments.

The colour intensity of the supernatants was also increased by increasing concentrations of ammonium chloride up to 100 μg./ml. Calcium chloride had a comparable effect. No other salts were found to stimulate pigment production although the following were tried: sodium chloride, potassium chloride, ammonium sulphate, ammonium citrate, sodium sulphate, sodium phosphate (dibasic), sodium acetate, potassium phosphate (monobasic), magnesium sulphate, manganese sulphate, ferrous sulphate and zinc sulphate. The heavy metal salts were tested at concentrations of 0·01 p.c. and the others at both 0·01 and 1 p.c. It appeared from these results that the ammonium chloride and calcium chloride either facilitated the release of pigment from the cells or stabilised them in the supernatant medium.

In the presence of isoniazid and ammonium chloride, pigment production was not improved by additions of sodium citrate, sodium acetate, glucose, glycerol, glycine, serine, adenine, uracil, asparagine or casein hydrolysate.

Pigment production was reduced at pH 6 compared with pH 7. Suspensions incubated with isoniazid at pH 8 showed more coloured bands in the chromatograms. Some of the pigments showed a shift in spectrum with pH for which the pK was 7·6. It is therefore probable that at pH 8 both forms were present.

It was often possible to see a colour change in the cells and supernatants at 6-8 hours. However, by concentration of larger volumes it was possible to detect pigment in the cells but not the supernatants at 2 hours and in both

at 4 hours. The compounds were not stable in shaken suspensions and beyond about 40 hours' incubation would decline and then disappear altogether.

Compounds which inhibited the uptake of isoniazid also inhibited the production of pigment. These were sodium cyanide, sodium azide and 2:4-dinitrophenol (Youatt, 1958b) and nicotinic, picolinic and benzoic acid hydrazides (Youatt, 1960a). The chelating compounds, oxine, versene and o-phenanthroline were without effect on pigment production.

Description of pigments.

Four compounds were produced regularly and in sufficient amounts to be purified to a stage where they gave reproducible spectra.

Compound 1. This was a yellow, non-fluorescent pigment. The ultra-violet spectrum is shown in Fig. 1. The compound was unstable when purified and Fig. 1 also shows the spectrum after storage of the solution in the deep-freeze for two days. The presence of inorganic salts probably had a stabilising effect because the stability of the compound was notably diminished in the final stage of separation when the pigment was dissolved in absolute ethanol to remove the small remaining traces of inorganic salts. Individual compounds were not tested for light stability but the colour of the whole supernatant was lost in sunlight and consequently procedures were carried out in the dark as far as possible.

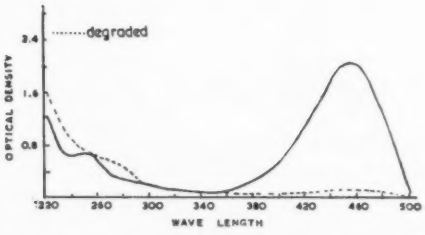


Fig. 1. Ultra-violet absorption spectrum of Compound 1 in distilled water with its degradation product after storage. Wavelength in mμ. Molar extinction coefficient of this sample at 455 mμ. was 5200.

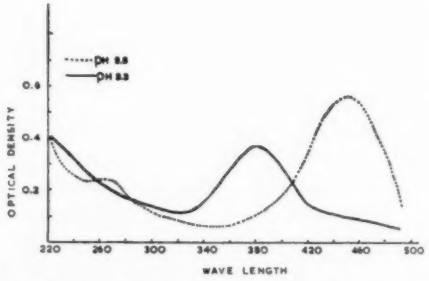


Fig. 2. Ultra-violet absorption spectrum of Compound 1 at pH 8·8 and 3·3 showing shift in absorption maximum. Wavelength in mμ.

Fig. 2 shows the shift in ultra-violet spectrum with change in pH. The pK for this change was found to be 7·6.

The infra-red spectrum of this compound in a KCl disc is shown in Fig. 3. Considerable difficulty was involved in the measurement of the infra-red spectrum as the compound was hygroscopic and insoluble in any of the recommended solvents.

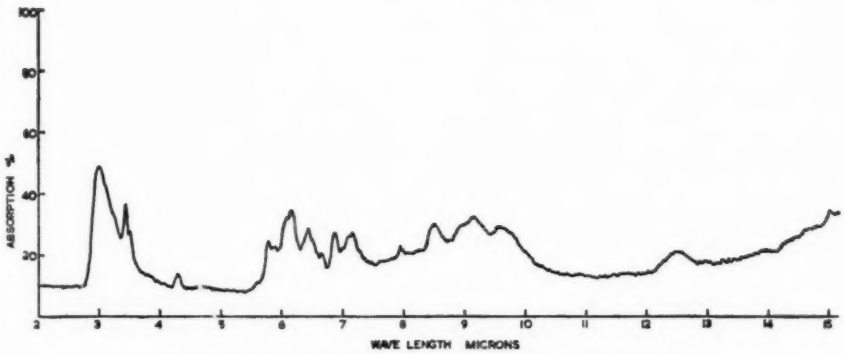


Fig. 3. Infra-red spectrum of Compound 1 in a KCl disc.

A maximum yield of about 5 mg. of pigment from 100 gm. dry weight of cells was obtained. In addition to the difficulties of working with small amounts of material, investigations were hampered by its instability and the failure to obtain either the compound itself or a derivative in a crystalline state.

Reaction with acetyl chloride, methyl iodide and diazomethane resulted in the breakdown of the compound. With chloroplatinic acid an amorphous precipitate was formed which was highly insoluble and could not be recrystallised. On addition of picric acid an amorphous, hygroscopic precipitate was formed. With 2:4-dinitrophenylhydrazine a hydrazone was precipitated slowly from the pigment. If the pigment was first treated with bromine an immediate precipitate was found on addition of 2:4-dinitrophenylhydrazine. Neither hydrazone could be crystallised. Reduction with hydrogen in the presence of Adam's catalyst gave a white solid with an ill-defined ultra-violet spectrum, shown in Fig. 4. The compound did not sublime *in vacuo* and decomposed on heating *in vacuo* above 100° C.

The compound was therefore purified as far as possible using the ultra-violet absorption spectrum as a criterion of purity. The molar absorption coefficient at 455 mμ of the best samples was 5,200. Analysis then gave C, 48·8; H, 6·2; N, 10·9 p.c. The molecular weight by isothermal distillation was 132. Two molecules of hydrogen were taken up for each molecule of pigment in the presence of Adam's catalyst. Fehling's solution, Benedict's solution and silver nitrate were reduced by the pigment which was itself irreversibly reduced by dithionite, when a fleeting red colour was often observed.

It is not possible to draw any conclusion about the identity of the compound. It appears to be a small molecule with one or more carbonyl groups. The acid-base shift in spectrum is such as might be observed with a Schiff's base. The compound is extremely soluble in water and ethanol, but insoluble in most organic solvents. When isolated from a chromatogram developed in

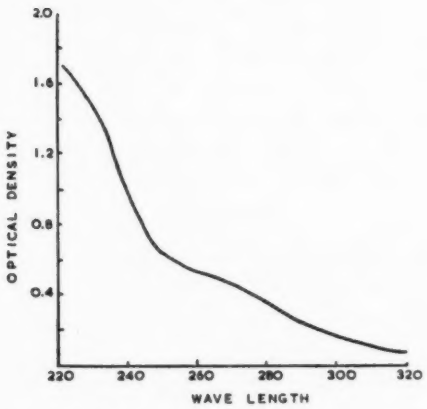


Fig. 4. Ultra-violet absorption spectrum of Compound 1 after hydrogenation in the presence of Adam's catalyst.

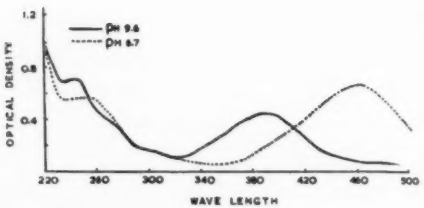


Fig. 5. Ultra-violet absorption spectrum of Compound 2 at pH 9·6 and 6·7, showing shift in absorption maxima with pH. Wavelength in mμ.

acid solvent it was insoluble in butanol but dissolved on the addition of ammonia. These observations suggest that it is a weak base. As the pigment is less stable in alkaline solution it is probable that the base is less stable than its salts. It appeared less probable that the compound was a weak acid capable of forming an ammonium salt which was soluble in butanol.

Compound 2. This was a red non-fluorescent pigment with the ultra-violet spectrum shown in Fig. 4. There was a shift in the spectrum with pH similar to Compound 1. This is shown in Fig. 5. This compound was particularly unstable in solution and was always contaminated by its degradation product which was a weakly yellow fluorescent material. It was also more difficult to separate from inorganic salts and has not been examined in detail. There appears to be a very close relationship with Compound 1 and it showed the same oxidising and reducing abilities.

Compound 3. This was a yellow compound with a yellow fluorescence under ultra-violet light. It was obtained by extraction of the cells after exposure to isoniazid, with boiling distilled water. The ultra-violet spectrum is shown in Fig. 6 which also shows its acid-base shift. This compound appeared to be more stable than 1 and 2 but was not obtained in sufficient amounts because of the difficulties of working up the boiled cell extracts.

Compound 4. Fig. 7 shows the spectrum of another yellow fluorescent compound. There was no acid-base shift with this compound. It was reversibly reduced by dithionite and its fluorescence was quenched by strong alkali. The R_F in several solvents was the same as the R_F of a known sample of riboflavin.

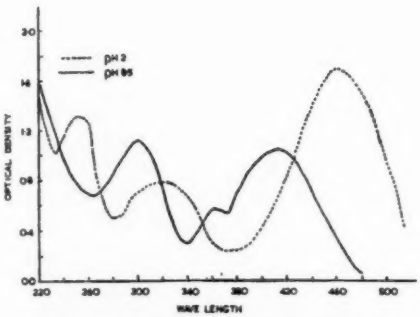


Fig. 6. Ultra-violet absorption spectra at pH 8·5 and 2 for Compound 3. Wavelength in mμ.

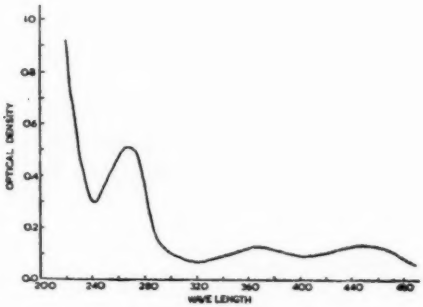


Fig. 7. Ultra-violet absorption spectrum of Compound 4 in distilled water. Wavelength in mμ.

These observations suggest that the compound is riboflavin or some closely related compound.

Compound 4 was produced under the conditions given for the production of pigments but experimental conditions could be modified to give this compound as the main pigment produced. In the absence of ammonium chloride and with 20 μg./ml. isoniazid, Compounds 1 and 2 were not produced in the supernatants, although they were present in the cells. In suspensions of B.C.G. shaken in air for 24 hours before the addition of isoniazid, Compound 4 was produced without Compounds 1 and 2.

The origin of the pigments. Attempts have been made to determine whether the pigments are derived from the isoniazid or are a product of disordered cell metabolism. The use of randomly labelled C^{14} isoniazid would have given direct evidence but unfortunately this was not available. The alternative indirect methods have favoured the view that the pigments are derived from the cell but they are not conclusive. The pigments were not radioactive when prepared with suspensions exposed to isoniazid labelled in the carbonyl group but this did not eliminate the possibility that the pyridine ring was present. 2-methyl and 2-ethyl isoniazid were tested in the hope that the pigments, if derived from them, would be chromatographically or spectroscopically distinct from those produced in the presence of isoniazid. Pigment production was good with 2-methyl isoniazid and barely detectable with 2-ethyl isoniazid but the pigments produced were not distinguishable from those produced in the presence of isoniazid. When the cells were starved by shaking them in air for 24 hours before the addition of isoniazid, Compounds 1 and 2 were not produced although isoniazid was still metabolised.

Consideration was also given to the possibility of finding sufficient discrepancy between the loss of isoniazid and the products of its breakdown to account for the pigments produced. From the molar extinction coefficient of

Compound 1 it was determined that the average supernatant contained about 5 μg./ml. after the cells had been incubated with 200 μg./ml. isoniazid. The pigment concentrations therefore lay within the experimental errors of the estimations.

In conclusion, a clearer view of the action of isoniazid may be obtained by a summary of the sequence of events when cells are exposed to isoniazid. Isoniazid is at once taken up by sensitive cells and saturation level is nearly reached in 2-4 hours (Youatt, 1958b). At 2 hours the products of isoniazid breakdown, *iso*nicotinic acid and 4-pyridyl methanol are detectable in the cells. Compounds 1 and 2 are also detected. From 4 hours onwards both the metabolic products of isoniazid and the pigments appear in the supernatants.

With resistant suspensions little uptake is observed on addition of isoniazid. In the cells only *iso*nicotinic acid is found and no pigments are produced.

The following mechanism is suggested to account for the sequence of events described above. Isoniazid is taken up by sensitive cells and combines with certain carbonyl compounds which are essential intermediates in the cell metabolism. These hydrazones are broken down in the cell to yield 4-pyridine aldehyde and Schiff's bases of the carbonyl compounds. 4-pyridine aldehyde has been shown to be metabolised rapidly by the cells with the formation of *iso*nicotinic acid and 4-pyridyl methanol (unpublished data).

Any theory to account for the mode of action of isoniazid must also consider the observed differences in the behaviour of sensitive cells when exposed to nicotinic acid hydrazide. It will, therefore, be necessary to isolate and identify the products of the breakdown of nicotinic acid hydrazide, in particular the compound which has an R_F like 4-pyridyl methanol (unpublished data).

Acknowledgments. This work was supported by the National Health and Medical Research Council of Australia. I wish to thank Professor S. D. Rubbo for his continued interest and encouragement, and Dr. R. G. Cooke of the Department of Chemistry, University of Melbourne, for much helpful advice.

REFERENCES.

Youatt, Jean (1958a): Amer. Rev. Tuberc., 78, p. 806.
Youatt, Jean (1958b): Austral. J. exp. Biol., 36, p. 223.
Youatt, Jean (1960a): *Ibid.*, 38, p. 331.
Youatt, Jean (1961): Austral. J. Chem., Vol. 14 (in press).

ANALYSIS OF MAJOR HISTOCOMPATIBILITY FACTORS IN A STOCK OF CLOSELY INBRED WHITE LEGHORN FOWLS USING A GRAFT-*versus*-HOST REACTION ON THE CHORIOALLANTOIC MEMBRANE

by DEBORAH BURNET AND F. M. BURNET

(From the Walter and Eliza Hall Institute of Medical Research, Melbourne).

(*Accepted for publication 20th December, 1960.*)

SUMMARY.

A study of two sublines of a closely inbred White Leghorn stock has been made using the number of foci produced after transfer of adult leucocytes to chorioallantoic membranes as an indication of the degree of genetic difference between donor and embryonic host.

If minor degrees of reactivity are neglected for the time being, the results are explicable in terms of three alleles, A, B, C, at a single locus, and following clonal selection theories of immunity, on the assumption that each fowl carries leucocyte cell clones capable of reacting with antigens not present in its body.

INTRODUCTION.

Several papers from this laboratory have been concerned with a new and convenient way of demonstrating the Simonsen phenomenon by depositing blood or leucocytes from an adult fowl on the chorioallantoic membrane (CAM) of suitable chick embryos. In a preliminary communication (Burnet and Burnet, 1960) we have described the general character of the influence of genetic factors in inbred lines on the production and numbers of immune foci on the CAM. In the present paper we give a more complete description of the findings obtained with the same two stocks of inbred fowls.

The production of proliferative foci on the chorioallantois is a manifestation of Simonsen's phenomenon and is undoubtedly a graft-versus-host immunological reaction (Simonsen, 1957). The effective agent in adult blood is cellular and our own experience is in line with Terasaki's (1959) contention that lymphocytes are responsible. Approximately one focus on the CAM is produced per 20,000-40,000 leucocytes, i.e. in round figures one per 10^4 lymphocytes. It is likely that each focus is initiated by a single cell rather than by aggregates of inoculated cells, but the distribution of counts on a large series of membranes does not give a normal Poisson distribution and no other way of proving this assumption has yet been found. The histology of the lesions has been described (Burnet and Boyer, 1960) and following their interpretation

we believe that the essential process is an interaction between the immunologically mature cell (or its descendants) and foreign antigenic determinants in the embryonic tissues. As a result, adjacent cells are stimulated to more or less uncoordinated proliferation. We believe, largely on the basis of Biggs and Payne's (1959) finding, that both donor and host cells multiply actively in the lesion. The essential features of the reaction are: (1) immunologically mature leucocytes are necessary; normal embryonic blood as late as 19 days' incubation is inactive, and (2) there must be a genetic (antigenic) difference between the donor of the leucocytes and the reacting embryo. For reasons which will be elaborated in the discussion we believe that the active cells are "normal" in the sense that there is no reason whatever to believe that they are cells which have been stimulated directly or ancestrally by the antigenic determinants with which they react.

In attempting to develop a consistent genetic interpretation of our results we have had to make some primary assumptions. In the first place, we have assumed that antigenic differences could be regarded as being directly determined by gene differences. It is legitimate, therefore, to use the same symbol for an allele as for the antigenic determinants that it sponsors. In view of the major significance of the H2 locus in mice, we have adopted as the simplest hypothesis that three alleles, A, B and C, at one locus are sufficient to determine the relevant antigenic characters within the lines we are dealing with. We assume also that each allele is responsible for the corresponding antigenic determinant and where we have a heterozygote both antigenic determinants are produced in the cells.

Since the CAM foci are initiated by cells, presumably by individual cells, we have made the assumption based on the clonal selection theory of immunity that the cells concerned differ from the majority by possessing a preformed specificity for an antigenic determinant present in the host embryo but *not* in the donor fowl. By hypothesis each fowl will possess a proportion of immuno-

TABLE 1.

Implications of the hypotheses used.

Genotype of donor	Cells	Foci on embryos of genotype					
		AA	AB	AC	BB	BC	CC
AA	b c	−	++	++	++	++	++
AB	c	−	−	++	−	++	++
AC	b	−	++	−	++	++	−
BB	a c	++	++	++	−	++	++
BC	a	++	++	++	−	−	−
CC	a b	++	++	++	++	++	−

logically competent cells capable of reacting with all of the A, B, C antigenic determinants not represented in its own constitution.

If we have an adult fowl with the constitution AA, it will possess (amongst many others) clones of lymphocytes, represented as b, which are capable of reacting with antigenic determinant B, and others, represented as c, reactive with C. Blood from this fowl will therefore produce foci on embryos of all constitutions other than AA. Table 1 which, it should be stressed, is at this stage purely hypothetical, should help to clarify the general pattern of results to be expected on our hypothesis.

Materials and Methods.

Origin of pure lines.

The Poultry Research Centre of the Commonwealth Scientific and Industrial Research Organization (C.S.I.R.O.) was set up in 1946 and in 1948 three subpopulations of White Leghorn pullets were selected for inbreeding and the development of certain characters, including intensity of laying (Skaller, 1957). Each subpopulation derived from a single brother-sister mating and close inbreeding of these lines has been practised for 10 generations. One of these, strain B1, has been the source of all our stock during the past two years. Two separate sires were used to initiate our sublines I and II, the birds in question being grandparents of our 1960-61 stocks.

Over the seasons 1959-60 and 1960-61 we have tested progeny of these two sublines. The greater part of the work to be reported concerns the 1960-61 stock which has been available to us in the form of several groups of pullets, full sisters, mated to a full brother.

In subline I there are five groups with the pedigree shown—

Parents	4291 x 3726	4291 x 3756	4291 x 3808	4291 x 3809	4291 x 3857
of	I 1 ♂ 103	I 2 ♂ 102	I 3 ♂ 101	I 4 ♂ 108	I 5 ♂ 104
Mated with			149*		
full sisters	9 ♀	3 ♀	11 ♀	6 ♀	7 ♀

* Owing to poor fertility of eggs sired by 101, he was replaced by full brother 149 of the same AA genotype.

and in subline II, four groups as below—

Parents	4290 x 6558	4290 x 6563	4290 x 6568	4290 x 6569
of	II 1 ♂ 107	II 2 ♂ 134	II 3 ♂ 106	II 4 ♂ 105
Mated with				
full sisters	3 ♀	2 ♀	8 ♀	5 ♀

Methods.

Donor fowls were bled from a wing vein into one-fifth the volume of a solution of heparin in saline (100 units/ml.). The blood was spun at low speed and the superficial cellular layer transferred to capillary tubes. These were again centrifuged and the leucocytic layer resuspended in physiological saline containing 100 units/ml. penicillin and 500 μg./ml. streptomycin. Such cells can be kept up to 48 hours at 4° C. without loss of lesion-producing capacity.

The technique of inoculation has been described previously (Burnet and Boyer, 1960). It involves depositing 0·1 ml. of leucocyte suspension on the base of an artificial airspace with the egg rotated so as to exclude from the area inoculated the region immediately beneath the site of drilling. Eggs are then reincubated at 37° C. for four days. The mem-

branes are removed, washed free of blood in formol-saline and spread out for counting on a glass plate.

Similar results are obtained if heparinized blood is used directly. The main reason for the preliminary separation was to make it possible to count the leucocytes more satisfactorily in a hemocytometer. This was done routinely to obtain an estimate of the average number of cells needed to initiate each focus.

In general, cells from a bird of one of the pure line groups were inoculated on a series of embryos from the same subline or when required from the other subline. All eggs came from birds housed in individual cages and were labelled as they were collected. For obvious reasons it was only occasionally possible to use as many embryos of a given parentage as might be desired, but eventually we could obtain a definite genetic diagnosis for almost every pullet of the groups being studied that laid more than two or three fertile eggs. In addition to being tested on inbred embryos, each inoculum was placed on a control series of eight to twelve embryos obtained randomly from a commercial cross-bred (Australorp x White Leghorn) flock. The median count obtained on these control embryos was used as a basis for the evaluation of the results on the inbred embryos according to the scheme:

++	> 50 p.c. of the median count
+	20-49 p.c.
±	3-19 p.c.
−	0-2·5 p.c.

Genetic terminology.

As the work has progressed over the past two years, provisional nomenclatures have been used and modified according to the findings. In an earlier paper, the data obtained in the 1959-60 season were interpreted on a model which postulated that there were three alleles at one locus, ABC, and three at a second locus, DEF. The corresponding loci in subline II were designated OPQ, RST. It was postulated that subline I was predominantly AADD and subline II PPRR. With the recognition of some interaction between the two sublines, it was possible, as indicated earlier, to simplify the model and consider only one locus. It also seemed expedient to neglect for the time being evidence of minor factors shown by low counts given in certain combinations. Most of the birds in subline I are now regarded as AA, most of those in II as CC.

RESULTS.

Detailed records of all counts on individual inbred and random control eggs are available, but their full publication would be unnecessarily cumbersome. The general procedure can be exemplified by giving one set of typical results from the titration of leucocytes from fowl 904 ♀ subline I 3 on 15 embryos of subline I.

From this protocol it is evident that Group 5 is segregating for two factors and if the donor 904 is AA with b and c leucocytes, one of the factors B or C is present in Group 5. Further analysis of the situation is best done by considering the sublines separately and then in regard to their interaction.

Subline I. This has shown remarkable homozygosity; all the pullets appear to have the constitution AA as have four of the five cockerels. The cock in Group 5 (♂ 104) is, however, AB and when mated with his AA sisters should

TABLE 2.

Typical titration protocol: leucocytes 904 (*AA*) *on subline* 1 *embryos*

Parentage of embryo ♂ ♀	Group	Counts (Inoculum $5{\cdot}6 \times 10^6$ leucocytes/ml.)	
		No. of foci	Scored as
102 × 8	I 2	0	−
149 × 586	I 3	0	−
108 × 496	I 4	1	−
108 × 690	I 4	0	−
108 × 4414	I 4	0	−
108 × 4550	I 4	1	−
104 × 288	I 5	1, 0	− −
104 × 375	I 5	0	
104 × 485	I 5	56, 38, 0	++, ++, −
104 × 678	I 5	100, 0	++, −
104 × 4444	I 5	2	−
Random controls		35, 58, 60, 51, 63, 77, 100	
Median		60	

give equal numbers of AA and AB embryos. These can be differentiated by the production of foci by AA blood (containing b cells) on AB embryos but not on AA embryos. Table 3 shows the number of ++ and − or ± results obtained when AA blood was tested on the progeny of ♂ 104. The ratio is not significantly different from 1 : 1.

TABLE 3.

Results obtained following inoculation of AA leucocytes on embryos from I 5 *group.*

	No. of embryos with ++ counts	No. of embryos with − counts
AB × AA, i.e. 104 × 288	8	9
× 375	7	9
× 485	9	3
× 678	9	8
× 4444	9	5
Total	42	34

Subline II. This line is less homogeneous than I, but there is a predominance of individuals homozygous for C. Such will by hypothesis have leucocytes antiA and antiB (a, b). In group II 4 the cock ♂ 105 is CB while the sisters to which he is mated are CC. When the embryos of this group are tested with cells from CC birds, again the ratio 10 : 16 is not significantly different from 1 : 1.

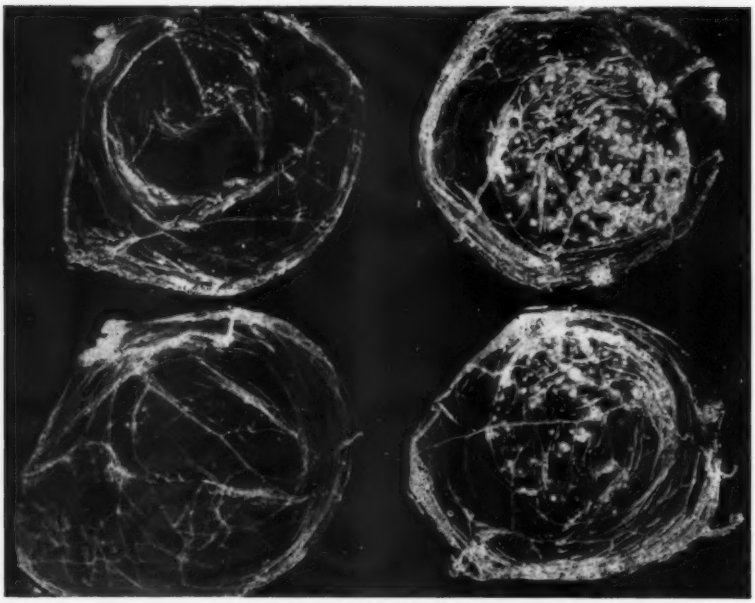


Fig. 1. *To show segregation of antigen B in CC x CB cross.* Leucocytes from 106 (CC) were tested on membranes of four embryos, all from eggs laid by 4525 (CB) after artificial insemination by the cock 106 (CC) providing the leucocytes. Counts of foci 116, 203, 0, 0.

TABLE 4.

Results on embryos from CC×CB matings when tested with CC(a, b) leucocytes.

Embryos	No. of embryos with counts shown	
	++	− or ±
♂ 105×583	1	3
×694	6	7
×1079	3	0
×4442	0	6
Total	10	16

A fowl with CB genotype should carry antiA cells only and such leucocytes should be capable of detecting the presence of A in any embryos produced in subline II. Leucocytes from ♂ 105 were used with eggs produced by birds from each group in the subline. Results from matings providing two or more embryos are shown in Table 5.

TABLE 5.

Tests on selected embryos for the presence of antigen A using leucocytes of a CB donor.

Embryos ♂ ♀	No. of embryos with counts shown	
	++	− or ±
II 1 107×187	2	2
II 2 106×4385	−	2
II 3 134×1029	3	2
II 4 105×1079	−	2

This indicates the A allele is segregating in (1) and (3) and since from other evidence ♂ 107 is CC, then 187 must be CA, giving offspring CA : AA ratio 1 : 1 as observed. The fact that 1029 gave disproportionately high number of ++ when tested on subline II embryos led to its being tested on subline I embryos. The results were consistently negative, indicating that in fact it was AB. 1029 was mated with 134 which was CA, thus allowing four types of possible offspring.

Cross reactions between sublines I and II.

Results of cross-testing have conformed to the genotype designation – the bulk of homozygous fowls give full ++ positives when tested on embryos of the opposite line, but with the heterozygotes different patterns of reactivity on the opposite line are obtained. Subline II bird 134 (CA) leucocytes gives

TABLE 6.

Presumptive genotypes of the 1960-61 *chickens of the inbred stocks.*

	Subline I				
	I 1	I 2	I 3	I 4	I 5
Cocks	103 AA	102 AA	149 AA	108 AA	104 AB
Pullets	9 AA	3 AA	5 AA	5 AA	6 AA
			(6)	(1)	(1)
	Subline II				
	II 1	II 2	II 3	II 4	
Cocks	107 CC	134 CA	106 CC	105 CB	
Pullets	3 CA	1 AB	6 CC	3 CC	
		1 CB	2 CB	1 CB	
				(1)	

Figures in brackets indicate the number of pullets in the group for which there is insufficient evidence to give the genotype.

– on all subline I embryos except that group with AB cockerel where both ++ and – are observed.

The result of testing both leucocytes and embryos of the two sublines has enabled us to determine the genotype of almost every bird at least as far as one major gene locus is concerned.

Table 7 gives a summary of all our results arranged after presumptive genotypes had been allocated.

TABLE 7.

Summary of all focal counts results from tests within sublines I and II.

Leucocyte donor genotype	Embryonic host genotype	Results (Number of tests performed)			
		++	+	±	–
AA (bc)	AA	–	14	83	155
AA (bc)	AA, AB*	43	1	8	25
	(AB)	(43	1	–	–)
AA (bc)	CC, CB	33	2	1	–
AB (c)	AA	–	1	6	24
AB (c)	CC, CB, CA	12	3	–	–
CA (b)	AA	–	3	8	19
CB (a)	CC, CB	–	7	11	33
CB (a)	AA, AB	44	2	–	–
CC (ba)	CC	–	–	7	44
CC (ba)	CA	4	–	–	–
CC (ba)	CC, CB*	12	1	3	17
	(CB)	(12	1	–	–)
CC (ba)	AA, AB	48	1	–	–

* It is presumed that AB and CB embryos present amongst these two groups were responsible for the results shown in parentheses.

The + and ± results presumably indicate the presence of minor histocompatibility factors but it will be seen that if only ++ and – results are considered there are no exceptions to the results expected. Further breeding experiments are in progress in an attempt to determine the nature of the minor interactions.

DISCUSSION.

The main interest of these results, apart from their providing what may be a useful method for the control of pure line breeding in fowls, is in their possible relevance to the nature of immunological reactivity. It is clear from the results that a very small proportion of the leucocytes placed on a chorio-allantoic membrane differing in genetic-antigenic makeup from the donor of the leucocytes will give rise to foci. If, as seems highly probable, the effective cells are the lymphocytes and other mononuclear cells of the blood, the median

counts are usually of the order of one focus per 10^4 lymphocytic cells. On genetically identical membranes no foci are produced. Since in several protocols we have observed sharp segregation of results within full sibling embryos inoculated with the same leucocyte suspension, it is evident that a single gene difference can result in an embryo showing a full count of foci or none at all.

Possible immunological interpretations can be discussed in a series of binary choices.

1. The cells which initiate foci differ specifically from those which do not, *or* all lymphoid cells are potentially capable of producing foci but owing to various essentially accidental factors only 1 in 10,000 actually achieves initiation of a focus.

2. Only cells preadapted to do so can react with the foreign antigen and in virtue of this initiate a focus *or* each cell is capable of differentiating between a CAM antigenically equivalent to itself and one which has one or more foreign antigens.

3. Recognition of foreignness by the effective cells is due to their possession of a preformed antibody-like receptor which can react with and be stimulated by the foreign antigenic determinant *or* the effective cells have no immunological reactivity until following contact with the foreign antigenic determinant, they develop an appropriately patterned immunological mechanism by what Lederberg has called an "instructive process".

4. Preadaptation is due simply to genetic (including somatic genetic) processes *or* it results from stimulation in the donor by the corresponding antigenic determinant.

The general trend of the results falls neatly into the pattern of a clonal selection theory which accepts the first of each of the above pairs of alternatives. It is probably impossible, however, to make any of the four choices on unequivocal experimental evidence. Theoretical interpretations must be chosen therefore primarily on a basis of convenience and ability. Our main difficulty in interpreting the results in terms of any of the current instructive theories of immunity is the absence from such theories of any approach to the problem of non-antigenicity of self-components. Until this is remedied discussion of phenomena in this general field will necessarily have to be couched in "selectionist" terms.

Acknowledgments. This work was only possible as a result of the close and friendly cooperation of Dr. J. A. Morris and his technical staff at C.S.I.R.O. Poultry Research Centre, Werribee. We are deeply indebted to them for their active help and to Dr. J. M. Rendel, Chief of the Division of Animal Genetics, C.S.I.R.O., for permission to use these facilities and for his interest in the problem.

The excellent technical assistance of Mrs. Rae Stranger and Miss Jill Cumpston is acknowledged.

REFERENCES.

Biggs, P. M., and Payne, L. N. (1959): Nature, 184, p. 1595.
Burnet, F. M., and Boyer, Georgiana (1960): J. Path. Bact. (in press).
Burnet, F. M., and Burnet, Deborah (1960): Nature, 188, p. 376.
Simonsen, M. (1957): Acta Path. Microbiol. Scand., 40, p. 480.
Skaller, F. (1957): Technical Paper 2, Div. Animal Health and Production, C.S.I.R.O.
Terasaki, P. I. (1959): J. Embryol. exp. Morph., 7, p. 394.